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BACTERIOLOGICAL STUDIES OF FIELD SOILS

III. THE EFFECTS OF BARNYARD MANURE

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SUMMARY.

The conclusions which may be drawn from the experiments upon the effects of barnyard manure on bacteriological activities of field soils presented in this bulletin are as follows:

1. Applications of manure up to sixteen tons per acre increased the numbers of organisms in the soil as shown by the growth on modified synthetic and albumen agar. The ammonifying power of the soil as shown by tests with the casein-fresh soil, albumen-fresh soil, dried blood-fresh soil, and dried blood-air-dried soil methods, and the nitrifying power tested by the ammonium sulfate-fresh soil, and the ammonium sulfate-air-dried soil methods were likewise increased.

2. The greatest increases occurred between the check soil and that receiving eight tons per acre and between the soil receiving the eight tons and that to which twelve tons per acre were applied. In most cases only a very slight increase occurred in the soil on which sixteen tons were used over that where twelve tons were added.

3. Twenty tons of manure per acre caused a depression in numbers of bacteria, in ammonifying power, and in nitrifying power according to all the methods employed, the results being lower than those secured when twelve tons per acre were added.

4. Albumen agar permitted of the development of larger numbers of soil organisms than the modified synthetic agar and also permitted of a greater differentiation between the soils of the various plots.

5. There was a close relationship between the ammonifying power of the soils and the numbers of organisms in them according to the methods used in this work.

6. The casein-fresh soil method of testing the ammonifying power of the soil was the simplest, permitted of the greatest differentiation between different soils, and in general was the most satisfactory.

7. The ammonium sulfate-fresh soil method for testing the nitrifying power of the soil showed the greatest differences between the various soils and is recommended as the more rational method.

8. The nitrifying power and the ammonifying power of the soil according to the methods used proceeded in the same direction.

9. Applications of manure up to sixteen tons per acre increased the yield of corn from the plots in this series, the great-

est increases occurring between the check plot and that receiving eight tons per acre and between the latter and the plot to which twelve tons per acre were added.

A very slight further increase occurred when sixteen tons per acre were applied.

10. Twenty tons of manure per acre depressed the crop yield below that obtained when twelve tons per acre were added.

11. The results of the bacteriological tests and the crop yields coincide almost exactly. Further evidence is thus supplied that there is a close relationship between bacterial activities and the fertility or crop-producing power of soils.

12. The depression in crop yields and bacterial activities caused by twenty tons of manure per acre cannot be attributed to denitrification as tests by the Giltay solution method and the soil method give no evidence of losses of nitrogen. The depression must therefore be due to physiological or other causes.

THE EFFECTS OF BARNYARD MANURE

BY PERCY EDGAR BROWN

The use of farm manure to increase crop yields was common more than two thousand years ago, but the reasons for such increase remained a mystery until the brilliant researches of Liebig in the middle of the last century. He showed that certain chemical elements were essential for the growth of plants and that manure contained varying amounts of these elements. It was believed then, therefore, that the beneficial effect of manure was due to the addition of these chemical plant food constituents to the soil. Now it is commonly recognized that farm manure exerts a fourfold effect on soils; not only a chemical, but also a physical, a bacteriological and a physiological effect. Knowledge of the last is still somewhat hypothetical and is based on the supposed toxic influence of organic substances on crops. The physical effects of manure as, for example, in opening up tight soils and rendering light, open soils more retentive of moisture and plant food, are too well known to need any discussion, as are also the chemical effects which consist mainly in adding plant food to the soil.

The bacteriological effects of manure, however, have not been extensively studied, although receiving some attention now in connection with the investigations in soil bacteriology, and no definite principles governing the action of manure on bacterial processes in the soil have yet been evolved. This is due to two reasons. First, the methods employed to test bacterial activities in the soil have been so unsatisfactory and so constantly changing that the results obtained by their use can hardly be regarded as showing the actual extent and importance of soil bacterial processes or as bringing out the true differences in soils due to the effects of different treatments. In the second place, results of tests of soils kept under the artificial conditions of the greenhouse or laboratory should not be considered as more than indicative of what may be occurring under actual field conditions. As has been pointed out by various writers, the air, moisture, and general climatic conditions exert such a vital effect on the bacteria in soils that unless these conditions are natural, the results of bacterial tests can hardly be considered applicable to field soils.

The many difficulties encountered in examining field soils were pointed out in the first work in this series, and also the means by which some of them may be eliminated or at least so far overcome as to make them negligible.

USE OF MANURE INVOLVES MANY QUESTIONS.

Many questions are involved in the use of manure on soils and considerable research will be necessary before a definite understanding of the principles concerned and the effects produced can be reached. There must be chemical study of soil organic matter and the organic constituents of manure; likewise, there must be study of the effects of the various compounds in different manures on important groups of bacteria and on various crops. In short, the problem is very complicated, so complicated that it would be clearly unwarranted, if not absolutely ridiculous, to say that the effects of manure are entirely physiological. It is just as unwarranted to claim that the chemical or the physical effects are of prime importance. The bacteriological changes brought about by manure must be considered fully for they are very largely dependent on its chemical and physical effects and may, therefore, be regarded as indicative of the trend of these other effects. No claim can be made, however, that the bacteriological changes are more vital than the others, but merely that they are of more significance. Physical and chemical effects of manure on crop yields are not brought about directly by physical and chemical means but indirectly by definite influence on bacterial activities. Furthermore, it is not too great an assumption to say that the physiological effects of manure, if there be such, are probably not caused entirely by physiological action on the plants but indirectly by physiological action on soil organisms.

It is not the purpose of these experiments, however, to undertake to solve the complicated problem of the various effects of manuring. Considerable work under a great variety of conditions will be necessary before anything approaching a solution will be possible. This work was begun merely to throw some additional light on the bacteriological phase of the problem from the field standpoint and also to secure additional data regarding the relation between bacterial activities and actual crop yields. Earlier work on field soils and some results still unpublished indicate the existence of a definite relation between certain bacterial processes in the soil and the crops produced on them, but much more data is necessary for any definite conclusion. Also, as will be noted later, new methods for the bacteriological examination of soils recently devised are to be tested and further evidence of their value secured.

HISTORICAL.

All experiments dealing with the effects of manure on the bacteria in the soil have shown not only a considerable increase in the numbers of organisms but also in the activities of certain groups of organisms particularly important from the fertility standpoint. The increase in actual numbers of organisms is to be expected when it is remembered that manure contains an enormous number of bacteria, variously estimated at from 7,000,000 to 375,000,000 per gram. Furthermore, the materials present in manure which serve as bacterial food naturally encourage the multiplication of organisms to a large extent.

Among the investigators reporting increases in numbers of organisms present in soil upon application of manure may be mentioned Caron[1], Remy[2], Fabricius and Von Feilitzen[3], Engberding[4], and H. Fischer[5].

In a more recent work Temple[6] showed that an addition of ten tons of cow manure per acre to a soil greatly increased the number of bacteria in that soil and that this increase continued over a considerable period. He found also that sterilized manure caused a greater increase than unsterilized. Thus it would seem that in this particular case the chemical or physical composition of the manure carried more influence than the bacterial content. Probably the sterilized manure encouraged the growth of a particular group of organisms which developed on the medium employed in the counts and to such a large extent that greater numbers were obtained than was the case when the manure containing its millions of living organisms was used. The possibility that the sterilization of the manure in the first case changed the chemical character of the manure, as well as the possibility of some toxic effects of the unsterilized material on the soil organisms, must be considered.

On the other hand Hellstrom[7] found that on moor soils fertilized with sterilized and unsterilized manure, the largest yields were obtained with the unsterilized material. He concluded that this effect was due to the bacteria added in the manure. Unfortunately, Temple did not have the crop yield to compare with his bacteriological results and Hellstrom did not determine numbers and activities of bacteria, so that we must attribute the discrepancy between the two results to the chemical and bacterio-

[1]Vortrag. geh. in. d. Wintervers.d. Zentralausschusses. d. Landw. gesellsch. Hanover., 26. XI. 1896 (ref. Jahresber. d. Garungsorg., 8, p. 212).
[2]Centbl. f. Bakt., Abt. II., Bd. 8, 1902, p. 734-762.
[3]Centbl. f. Bakt., Abt. II., Bd. 14, 1905, p. 165.
[4]Centbl. f. Bakt., Abt. II., Bd. 23, 1909, p. 601.
[5]Landw. Jahr., Bd. 38, 1909, p. 358.
[6]Bull. 95, Georgia Agr. Expt. Stat., 1911.
[7]K. Dandt. Akad. Handl. 33 (1899) p. 167-171.

logical differences in the soils and in the manure employed, which, of course, may be large.

Temple concluded also that additions of cow manure increased the ammonifying efficiency of most soils whether it was applied in a sterile or unsterile condition. The results of his ammonification studies, therefore, did not confirm the quantitative determinations. In the case of nitrification, however, while an increase in the nitrifying efficiency of most soils was shown, the greatest increase occurred when unsterilized manure was added. This greater effect of unsterilized manure was found to be due to the actual addition of nitrifying organisms. Niklewski[8] determined the number of nitrite-forming organisms in two samples of manure and found about 32,000 present per gram.

Wohltmann, Fischer and Schneider[9], and Moll[10] showed that peptone decomposition, nitrification, ammonia assimilation, and nitrogen fixation in soil were more or less strongly increased by additions of manure. Considerable increase in the peptone decomposing power of soil with applications of manure was likewise shown by Lipman[11]. Welbel[12] noted increased nitrate formation in soils receiving manure, and Wollny[13] showed a large increase in carbon dioxide production attributable, of course, to the large amount of organic material introduced and to the bacterial activities encouraged thereby.

It will not be necessary to cite the literature bearing on the subject of denitrification and nitrogen fixation as affected by manure, as the present experiments do not include work along these lines. Suffice it to say that nitrogen fixation has been shown to be increased when manure was applied to soils, and denitrification also. In the latter case, however, from the methods employed in making the tests, the results are very questionable and it is commonly believed now that unless excessive amounts of manure are applied together with a nitrate fertilizer there is little or no danger of volatilization of nitrogen by denitrification.

THE PLOTS EMPLOYED.

Five one-tenth acre plots which have been under experiment for five years were chosen for this work. The plots are located

[8]Centbl. f. Bakt., Abt. II., Bd. 26, p. 114.
[9]Journ. f. Landw., Bd. 52, 1904, p. 97-125.
[10]Beitr. Z. Biochemie d. Bodens. Diss. phil. Leipzig, 1909, p. 45-54.
[11]Rpt. New Jersey Agric. Expt. Stat. 27, 1906, p. 135.
[12]Russ. Journ. f. Expt. Landw., 4, 1903, p. 307; 6, 1905, p. 163.
[13]Landw. Vers. Stat., 36, 1899, p. 211.

on the Wisconsin drift area, the particular soil type being a Marshall loam. They are quite uniformly level. Prior to 1908 the land had been used for ordinary farming rotations and had received no special treatment. In 1908 a system of cropping according to the regular four year rotations of corn, corn, oats and clover was begun with a crop of corn. This was followed by oats in 1909 and this in turn by clover in 1910. In the fall of 1910 manure was aplied as follows:

Plot No.	Treatment.
1004—	Check
1005—	8 tons manure per acre
1006—	12 tons manure per acre
1007—	16 tons manure per acre
1008—	20 tons manure per acre

In the following year the corn crop suffered very severely from a continued drought, that on the plots receiving the manure more so than on the check plot. Evidently the manure exerted a depressing effect on the crop yields, due to the dry weather.

In 1912, however, a good season brought the yield of the second corn crop up to the normal and the beneficial effects of the manure became apparent.

The crop yields will be given later and comparisons between these and the bacteriological activities in the various plots will be made. The tests of the bacteriological activities include determinations of the number of organisms in the soils and of the ammonifying and nitrifying powers of the soils, several methods being used in these determinations.

Four samplings were made, the first on August 2, the second on August 15, the third on August 22, and the last on September 9. The results obtained at the different dates are quite satisfactory and in most cases the same differences between the various soils are evidenced.

THE METHOD OF SAMPLING.

The method of sampling employed in this work was the same as has been given in the previous reports of the study of field soils[14] and need not be described again here. The method continues to prove satisfactory and to justify the claims which have been made for it. It may be noted in this connection that samples are never drawn immediately following a rain or during a

[14] Centbl. f. Bakt., Abt. II., Bd. 35, 1912, p. 234; Research Bul. 5, Iowa Expt. Stat., 1912, p. 194.
Centbl. f. Bakt., Abt. II., Bd. 35, 1912, p. 218; Research Bul. 5, Iowa Expt. Stat., 1912, p. 203.

severe drought, as the conditions in the soil are then deemed to be too abnormal to permit of representative results. Several days should elapse before samples are drawn after a heavy rain to permit the soil to return to its normal moisture content, and to allow many of the organisms which have been washed down into the lower soil layers to return to the surface via the capillary moisture. As is known, there is considerable depression in numbers of organisms in a soil after a continued drought and species relationships are considerably altered. It is but natural, then, to expect that the differences in bacterial conditions in various plots may be very largely obscured by the death or inactivity of important species and considerable care should be exercised also in thoroughly mixing the soil in the area from which the samples are drawn in order to secure as complete uniformity as possible.

THE QUANTITATIVE DETERMINATIONS.

In the quantitative determinations two media were employed, the first being the "modified synthetic" agar used in the previous work in this series of studies of field soils, and the second an albumen agar which was devised by the writer and described in a recent publication[15]. Comparisons of these two media were given in the publication just referred to and the purpose of using them both in this experiment was to afford additional data regarding the value of the albumen agar which according to the previous results allowed of the development of much larger numbers of organisms than the modified synthetic agar.

The composition of the albumen agar is as follows:

1000 c.c. distilled water
0.5 gm. K_2HPO_4
0.2 gm. $MgSO_4$
0.1 gm. Egg Albumen
10.0 gms. dextrose
trace $Fe_2(SO_4)_3$
15.0 gms. agar

The only difference between this medium and the modified synthetic agar is that in the latter the albumen is replaced by 0.05 gm. peptone.

The usual method of making the plates was employed; that is, 100 grams of soil were shaken for five minutes with 200 c.c. of sterile water and dilutions made of 1-2,000; 1-20,000, and 1-200,000. Two 1 c.c. portions of each of these dilutions were plated, one with modified synthetic agar and the other with albumen agar. The plates were incubated for three days at room

[15]Centbl. f. Bakt., Abt. II., Bd. 38, 1913, p. 497; Research Bul. 11, Iowa Expt. Stat., 1913, p. 297.

temperature at the first date, for four days at the second, and for five days at the third and fourth dates, at the same temperature.

The results of these quantitative determinations are given in tables I and II and are calculated as usual as numbers of bacteria per gram of air-dry soil. The moisture content of the soils at the different samplings is given in table III. Glancing over this table we find very little variation in the moisture conditions in the various soils at the different dates, the largest difference at any one date being only two and one-half per cent.

The moisture conditions being so nearly uniform, the differences in bacterial numbers and activities in the various plots, as brought out by these experiments, must be attributed, therefore, to differences inherent in the soil or brought about by different treatments. Differences inherent in the soil may be very largely neglected in this case as the soil is uniform in composition and the plots are located on level, well-drained land. Hence differences in treatment must be regarded as the cause of varying bacterial activities.

RESULTS WITH MODIFIED SYNTHETIC AGAR.

Turning to table I, which gives the quantitative results with modified synthetic agar, we note that at every sampling the plots receiving eight tons of manure per acre showed greater numbers than the check plot, and the plot receiving twelve tons gave a still larger number. At two dates the maximum number of organisms seems to have been reached with this latter amount of manure and a slight depression occurred with sixteen tons, but at the other two samplings a slight increase was obtained with the larger amount of manure.

TABLE I. QUANTITATIVE DETERMINATIONS.

(Modified synthetic agar)

(Bacteria per gram of air-dry soil.)

Plot No.	Aug. 2	Aug. 15	Aug. 22	Sept. 9
1004	2,800,000	1,700,000	2,150,000	2,125,000
1005	3,143,000	2,750,000	3,081,000	2,950,000
1006	3,331,000	3,726,000	3,400,000	3,248,000
1007	3,162,000	3,713,000	3,500,000	3,151,000
1008	3,224,000	3,204,000	3,180,000	3,024,000

Any conclusion regarding the amount of manure causing the maximum increase in numbers of organisms would, therefore, hardly be justifiable. The plot receiving twenty tons of manure per acre in every case showed fewer bacteria than those receiving sixteen and twelve tons per acre, but more than that receiving eight tons per acre. If still larger amounts of manure had been employed it is a matter of doubt whether a further depression in numbers would have occurred—even perhaps below the check plot.

TABLE II. QUANTITATIVE DETERMINATIONS.

(Albumen agar)

(Bacteria per gram of air-dry soil.)

Plot No.	Aug. 2	Aug. 15	Aug. 22	Sept. 9
1004	3,153,000	6,500,000	4,550,000	2,875,000
1005	3,665,000	7,550,000	5,183,000	3,750,000
1006	3,894,000	9,496,000	6,500,000	4,315,000
1007	4,046,000	9,501,000	6,450,000	4,121,000
1008	3,741,000	8,932,000	5,098,000	3,951,000

TABLE III. MOISTURE IN SOILS.

Plot No.	Aug. 2 Per cent	Aug. 15 Per cent	Aug. 22 Per cent	Sept. 9 Per cent
1004	15.00	20.00	20.00	20.00
1005	14.75	20.00	19.50	20.00
1006	14.75	19.50	20.00	17.50
1007	14.50	19.75	20.00	17.50
1008	15.00	19.50	19.50	18.00

RESULTS WITH ALBUMEN AGAR.

When we examine table II. which gives the results obtained by the use of the albumen agar, we find that much larger numbers are shown. The difference in the incubation period of the plates at different dates which has been mentioned was hardly apparent when the modified synthetic agar was used. The maximum counts were evidently obtained in three days. With the albumen agar on the other hand the best results were secured in four to five days, which allowed time for the development of many colonies which in three days were probably too small to be visible. Greater differences in the numbers from the different plots were also shown where the longer incubation periods were employed.

Practically the same relations were observed here as were noted with the modified synthetic agar. The plot receiving eight tons of manure per acre showed larger numbers than the check plot and that receiving twelve tons per acre still larger numbers. Again the plot to which sixteen tons per acre were applied, at two dates showed slightly greater numbers and at the other two samplings slightly fewer organisms, the differences being very small. Where twenty tons of manure per acre were applied, just as with the other medium fewer organisms were shown than where twelve tons were used, but more than where eight tons were added.

The effects of the application of manure, therefore, up to a certain amount seemed to be to cause an increase in numbers of organisms developing on the modified synthetic and albumen agars. Beyond this maximum amount, which was apparently about sixteen tons per acre, there occurred a distinct decrease in numbers of organisms, fewer bacteria being found than in the soil receiving the twelve tons per acre.

The variation in the results at the different dates on the soils receiving the sixteen tons of manure make it impossible to fix the point at which the maximum effects from the manure were produced.

As has been noted already, it was not the intention in this work to examine into the causes for the results secured, consequently the reasons for the increase in organisms with applications of manure up to about sixteen tons per acre followed by a decrease when twenty tons were used may be merely suggested. The increase without doubt may be attributed primarily to the addition of large numbers of organisms, to the addition of a large amount of bacterial food, and to the presenting of more favorable physical conditions for the multiplication of the organisms present in the soil as well as those introduced.

The depression in numbers of organisms when twenty tons of manure per acre were applied may be due to one of two causes, either to the effect of the organic matter in the manure on the groups of organisms which develop on the modified synthetic and albumen agars, or to the fact that encouragement to such an extent was given to groups of bacteria which do not develop on these media that the other groups were restricted and this depression appeared in the counts while the gains in the other groups could not be determined. It would be interesting to continue this work, using larger amounts of manure and to ascertain how far this depression in numbers would go.

Comparing the results obtained in using the two media we find that greater numbers were obtained in every case with the albumen agar, the greatest difference being apparent when the

incubation period was four days which was apparently the optimum incubation period for albumen agar. Furthermore, greater differences between the numbers of organisms in the different plots were given by the counts on the albumen agar than by those on the modified synthetic agar. There are, therefore, undoubtedly organisms which develop on the albumen agar which refuse to do so on the synthetic agar. The advantages which were attributed to the albumen agar in the work already cited were therefore borne out by these results. The one objection to the medium which was mentioned in the same work may be noted here as being of little importance. This objection was the cloudiness of the medium, brought about by the coagulation of the albumen when the medium was sterilized. By shaking the tubes of agar thoroughly before plating, the coagulation was broken up quite completely and gave no difficulty in the counting as the colonies were readily distinguishable from the minute particles of coagulum.

THE AMMONIFICATION EXPERIMENTS.

In the experiments to determine the ammonifying power of the soil four methods were used. These were described in a recent publication[16], and they were employed in this series to obtain additional evidence of their value. Casein, advocated in the work just referred to as the most satisfactory material to be used as a measure of the ammonifying power of soils, and albumen, which also gave evidence of some value, were employed with fresh soil as a medium. The results obtained were compared with those secured by using dried blood with fresh soil and dried blood with air-dried soil inoculated with infusions of fresh soils.

Six 100-gram portions of fresh soil obtained as already described were weighed out in tumblers and thoroughly stirred with a sterile spatula. To two of these portions 10 c.c. of a casein solution, prepared by dissolving 100 grams of casein in 1000 c.c. of water containing 70 c.c. of normal NaOH, were added. To the second two, ten c.c. of a 10% solution of albumen, prepared by dissolving sterile albumen in sterile water, were applied. To the remaining two, five grams of dried blood were added and thoroughly mixed with the soil. The moisture content of the fresh soil was determined and sterile water was added to the samples to bring them up to the optimum for the soil, 70% being considered the optimum for the dried blood. They were then covered and incubated for varying lengths of time.

[16] Central. f. Bakt., Abt. II., Bd. 39, 1913, p. 61; Research Bul. 11, Iowa Expt. Stat., 1913, 385.

TABLE IV. THE AMMONIFICATION OF CASEIN.

Plot No.	Lab. No.	Aug. 2 Mgs. N.	Average Mgs. N.	Lab. No.	Aug. 15 Mgs. N.	Average Mgs. N.
1004	41	38.46		541	67.10	
	42	37.28	37.87	542	69.45	68.27
1005	43	46.70		543	72.99	
	44	47.09	46.89	544	74.16	73.57
1006	45	51.40		545	76.91	
	46	52.19	51.79	546	78.09	77.50
1007	47	51.01		547	78.09	
	48	52.97	51.99	548	78.87	78.48
1008	49	48.78		549	74.56	
	50	48.78	48.78	550	75.73	75.14

Plot No.	Lab. No.	Aug. 22 Mgs. N.	Average Mgs. N.	Lab. No.	Sept. 9 Mgs. N.	Average Mgs. N.
1004	1041	67.10		1241	52.58	
	1042	67.89	67.49	1242	50.62	51.60
1005	1043	73.38		1243	59.25	
	1044	72.20	72.79	1244	58.47	58.86
1006	1045	78.48		1245	63.32	
	1046	79.26	78.87	1246	66.32	66.32
1007	1047	79.26		1247	65.92	
	1048	79.06	79.16	1248	65.53	65.72
1008	1049	74.16		1249	60.03	
	1050	75.34	74.75	1250	60.82	60.42

Samples of soil from the various plots were air-dried and sieved and two 100-gram portions of each were weighed out in tumblers. Five grams of dried blood were added to each and thoroughly stirred in and 20 c.c. of infusions of fresh soils, obtained at the same time as those used in the other methods and prepared by shaking 100 grams for five minutes with 200 c.c. of sterile water, were added. The moisture content here was adjusted to the optimum with sterile water, 70% being again taken as the optimum for the dried blood. These samples were also covered and incubated at room temperature.

The distillation of all of these samples was carried on as usual, transferring to copper flasks, adding water, heavy magnesium oxide and a little paraffin, collecting the ammonia in standard acid and titrating against standard alkali.

The results obtained when using the casein are given in table IV and the summarized results in table V. At the first date of

TABLE V. THE AMMONIFICATION OF CASEIN.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	37.87	68.27	67.49	51.60
1005	46.89	73.57	72.79	58.83
1006	51.79	77.50	78.87	66.32
1007	51.99	78.48	79.46	65.72
1008	48.78	75.14	74.75	60.42

sampling the incubation period was only three days, while at the latter three dates it was four days and consequently larger amounts of ammonia were produced in the latter cases.

The main fact to be obtained from table IV is that the duplicate determinations agree remarkably well, in most cases the agreement being as close as could be expected in a chemical method. As will be noted later, this is a great advantage for the casein method.

MANURED SOILS SHOW GREATER AMMONIFYING POWER.

Turning to table V for a summarization of the results we find that the soils receiving applications of manure all showed greater ammonifying power than the check soil. The plot receiving twelve tons of manure per acre showed a higher ammonifying power than that receiving eight tons. The soil to which sixteen tons of manure per acre were applied, except in one case when a slight decrease occurred, gave a slightly greater production of ammonia than that where twelve tons per acre were used. The application of twenty tons of manure per acre, however, decreased the ammonifying power of the soil below that given by the soil receiving twelve tons per acre, but it remained somewhat greater than that of the plot where eight tons were added. The large amount of manure in this latter case evidently depressed the activities of the ammonia producing organisms. This depression was coincident with a depression in number of organisms and hence indicates that the media employed for counting the numbers of organisms permit of the development of the ammonifying species. The largest ammonia production occurring thus in the soil receiving sixteen tons of manure per acre, it is probably a safe assumption that the maximum ammonification would occur on this particular soil type with application of manure somewhere about sixteen tons per acre.

TABLE VI. THE AMMONIFICATION OF ALBUMEN.

Plot No.	Lab. No.	I Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	II Ammonia Mgs. N.	Average Mgs. N.
1004	61	17.27		561	53.76	
	62	17.27	17.27	562	54.15	53.95
1005	63	24.15		563	57.68	
	64	23.94	23.54	564	56.90	57.29
1006	65	26.29		565	62.78	
	66	27.08	26.68	566	64.35	63.56
1007	67	27.08		567	65.53	
	68	27.47	27.27	568	64.75	65.14
1008	69	23.94		569	58.86	
	70	24.72	24.33	570	66.13	59.64

Plot No.	Lab. No.	III Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	IV Ammonia Mgs. N.	Average Mgs. N.
1004	1061	57.68		1261	54.15	
	1062	54.15	55.91	1262	52.97	53.56
1005	1063	62.00		1263	57.68	
	1064	62.00	62.00	1264	58.47	58.07
1006	1065	67.89		1265	70.24	
	1066	69.85	68.87	1 66	68.67	69.45
1007	1067	67.89		1267	69.45	
	1067	70.24	69.06	1268	71.02	70.23
1008	1069	61.35		1269	67.49	
	1070	62.78	63.56	1270	69.06	68.28

Table VI contains the results of using albumen as the measure of the ammonia producing power of the soil. The samples at the first date were incubated only four days while at the later samplings six days was the length of incubation. Hence the ammonia production at the first date was very much less than at the later samplings and the differences between the various soils were smaller. Six days seemed to be the optimum period of incubation. The duplicate determinations agreed quite closely, in only one or two cases were there discrepancies of any size.

In table VII, which gives the summarized results with albumen, it may be noted that the increased ammonifying power produced in the soil by addition of manure was again evidenced. Again the application of twelve tons per acre caused a larger

TABLE VII. THE AMMONIFICATION OF ALBUMEN.

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
1004	17.27	53.95	55.91	53.56
1005	23.54	57.29	62.00	58.07
1006	26.68	63.56	68.87	69.45
1007	27.27	65.14	69.06	70.23
1008	24.53	59.04	63.56	68.28

increase than eight tons. Sixteen tons in every case here caused a slightly greater production of ammonia than twelve tons. The conclusion reached from the results obtained with casein are therefore confirmed; that is, it seems that manure applied to soil at the rate of about sixteen tons per acre caused the maximum ammonia production.

The larger amount of manure, twenty tons just as in the previous case, reduced the ammonia production below that from the soils receiving twelve and sixteen tons but it still remained larger than that from the soil receiving eight tons per acre.

These results again coincided very largely with the results of the quantitative determinations and further evidence was thus supplied that the media employed in the latter determinations permit of the development of the ammonia producing organisms to a very large extent.

The results obtained by the use of dried blood in fresh soil are given in table VIII and the summarized results in table IX. These samples were incubated for five days, which has been found to be the optimum period for the ammonification of dried blood.

The duplicate determinations here, as is always the case when dried blood is employed, did not agree very closely. Such a large amount of ammonia is produced, it is so difficult to mix the dried blood thoroughly with the soil, and the difficulty in distilling because of foaming is so great that close agreement of duplicates is always impossible. Examining the summarized results in table IX we find that the effects of the manure on the ammonia production were quite pronounced.

The soil receiving twelve tons of manure per acre showed greater ammonifying power than that receiving eight tons, which in turn gave a greater ammonia production than the check

TABLE VIII. THE AMMONIFICATION OF DRIED BLOOD.
(Fresh Soil)

Plot No.	Lab. No.	I Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	II Ammonia Mgs. N.	Average Mgs. N.
1004	21	68.28		521	85.94	
	22	65.53	66.90	522	82.01	83.97
1005	23	86.72		523	94.18	
	24	82.80	84.76	524	90.25	92.21
1006	25	90.64		525	102.81	
	26	82.01	86.32	526	109.87	106.34
1007	27	100.06		527	107.91	
	28	95.75	97.90	528	111.05	109.47
1008	29	87.90		529	101.24	
	30	85.54	86.72	530	90.25	95.74

Plot No.	Lab. No.	III Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	IV Ammonia Mgs. N.	Average Mgs. N.
1004	1021	74.25		1221	67.10	
	1022	72.20	73.57	1222	66.32	66.71
1005	1023	85.94		1223	71.42	
	1024	82.01	83.97	1224	69.85	70.63
1006	1025	96.53		1225	84.76	
	1026	101.24	98.88	1226	86.32	85.54
1007	1027	93.35		1227	86.32	
	1028	102.42	98.88	1228	83.58	84.95
1008	1029	89.07		1229	78.48	
	1030	85.94	87.50	1230	75.34	76.91

TABLE IX. THE AMMONIFICATION OF DRIED BLOOD.
(Fresh Soil)

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
1004	66.90	83.97	73.57	66.71
1005	84.76	92.21	83.97	70.63
1006	86.32	106.34	98.88	85.54
1007	97.90	109.47	98.88	84.95
1008	86.72	95.74	87.50	76.91

TABLE X. THE AMMONIFICATION OF DRIED BLOOD.
(Air-dry Soil)

Plot No.	Lab. No.	I Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	II Ammonia Mgs. N.	Average Mgs. N.
1004	1	72.59		501	113.80	
	2	88.29	80.44	502	109.87	111.83
1005	3	86.14		503	113.80	
	4	98.39	94.76	504	120.86	117.33
1006	5	102.02		505	127.92	
	6	98.10	100.06	506	134.59	131.25
1007	7	104.38		507	153.43	
	8	97.32	100.85	508	120.86	137.14
1008	9	97.32		509	118.11	
	10	94.18	95.75	510	139.69	128.90

Plot No.	Lab. No.	III Ammonia Mgs. N.	Average Mgs. N.	Lab. No.	IV Ammonia Mgs. N.	Average Mgs. N.
1004	1001	113.01		1201	114.19	
	1002	99.67	106.34	1202	91.43	102.81
1005	1003	105.16		1203	114.97	
	1004	113.79	109.47	1204	119.29	117.13
1006	1005	129.10		1205	132.81	
	1006	115.37	122.23	1206	122.04	127.92
1007	1007	129.10		1207	137.34	
	1008	118.90	124.00	1208	128.71	133.02
1008	1009	116.94		1209	121.64	
	1010	110.66	113.80	1210	123.61	122.62

TABLE XI. THE AMMONIFICATION OF DRIED BLOOD.
(Air-dry Soil)

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
1004	80.44	111.83	106.34	102.81
1005	94.76	117.33	109.47	117.13
1006	100.06	131.25	122.23	127.92
1007	100.85	137.14	124.00	133.02
1008	95.75	128.90	113.80	122.62

soil. Where sixteen tons per acre were applied, at two dates there was shown a still greater ammonifying power, and at the other two samplings practically identical figures were obtained as where twelve tons were used. We may conclude again, therefore, that applications of about sixteen tons of manure per acre bring the soil to its maximum ammonifying power. The twenty ton application, however, just as was the case when the casein and albumen were used, depressed the ammonifying power of the soil below that of the soils receiving twelve and sixteen tons per acre, but hardly down to that of the soil to which eight tons were applied.

The results secured by this method confirm very largely those secured in the other cases and therefore the same agreement with the results of the quantitative determinations is to be noted.

An examination of table X for the results secured by using air-dried soil wih dried blood and inoculations of fresh infusions shows that the agreement between the duplicate determinations was very poor although the averages give about the same difference between the various soils as were shown by the other methods. These differences in duplicates by this method are unavoidable for the reasons which have been discussed. These samples were all incubated for six days, which has been shown to be the optimum period for dried blood in air-dry soil.

In table XI the average results show the ammonifying power of the different soils by this method. The application of eight tons of manure per acre caused a decided increase in the ammonifying power of the soil, twelve tons, a greater increase, and sixteen tons a still further increase, which however, at two dates was very slight. The twenty tons again depressed the ammonifying power of the soil below that shown where twelve and sixteen tons were employed but not below that where eight tons were applied. These results coincided very satisfactorily with those secured by the other methods.

Again, the effect of applications of manure in increasing the ammonifying power of the soil is clearly shown, the maximum amount of ammonia being produced when about sixteen tons of manure per acre were used. Beyond sixteen tons there occurred a depression in ammonifying power similar to that shown by the other methods. Again the results corresponded closely with the results of the quantitative determinations.

FACTS THAT STAND OUT IN AMMONIFICATION RESULTS.

Considering these ammonification results as a whole several facts should be emphasized. In the first place the effect of applications of barnyard manure on the ammonifying power of the soil was quite definitely shown. The increases secured were

too great to be accounted for merely on the basis of the plant food actually added in the manure. Of course the physical factors were of some importance but in this case greater effects have been noted than could be ascribed to them.

It seems, therefore, from these results that the effects of manure may be very largely due to the influence on the bacterial activities. Whether this influence is due to the chemical, physical, or bacteriological composition of the manure or to its physiological effect on bacteria in stimulating their growth cannot be stated, but it is undoubtedly the case that the effect on crop growth may be traced directly to the effect on certain bacterial activities. The ammonifying power of a soil, representing as it does the production of available nitrogenous material for plant growth, must therefore be linked up closely with crop production. That is, unless conditions are exceedingly abnormal in a soil, it may be assumed that the activities of the ammonifying organisms are indicative of the crop-producing power of the soil.

In the earlier work in this series of studies of field soils, the actual crops produced and the ammonifying power of the soils as measured in the laboratory have been found to be very closely correlated. The crop yields from the plots used in these experiments will be given later and their correspondence with these ammonification results noted.

NUMBERS OF BACTERIA AND AMMONIFICATION CLOSELY RELATED.

In the second place evidence is supplied that numbers of bacteria and ammonification are very closely related. This is in accord with the results already secured and is of much interest from the fertility standpoint. As has just been stated, we know that its ammonifying power may be a very close measure of the fertility or crop-producing power of a soil. The numbers of organisms found by the use of the albumen and modified synthetic agars varied in the different soils exactly as the ammonifying power of the soils varied and hence it may be concluded that the estimate of the numbers of bacteria in the soils included at least the major portion of the ammonifying species, and furthermore, that a definite relation between numbers and crop production may be traced.

Finally, the fact that too heavy applications of manure may be made to a soil was quite definitely shown. When more than sixteen tons of manure per acre were used a depression in the ammonifying power of the soil occurred corresponding to a decrease in numbers. It is evident therefore that too much manure may be positively injurious. It will be shown later that the crop yields were also reduced by the large amount of manure.

FRESH SOIL-CASEIN METHOD PROVES BEST.

Comparing the results secured by the various methods for the study of ammonification, previous conclusions were borne out by the figures at hand.

The most satisfactory method was again found to be the fresh soil-casein method. The differences were more pronounced when it was used, the duplicates agreed the best and the least difficulty in distilling was encountered. When albumen was used the main difficulty appeared in the preparation of a sterile solution. This was so difficult that in view of the fact that the albumen possesses no advantage over the casein, the method cannot be recommended. When the fresh soil-dried blood-method was used, the amounts of ammonia produced were so large and so much carbon dioxide was formed that there was great difficulty in distilling. This fact, together with the difficulty in mixing the dried blood thoroughly with the soil, may be held accountable for the poor agreement of duplicates. When the dried blood was used with air-dry soil the same difficulties were experienced, and an additional objection to the method lies in the fact that air-dry soil is not sterile and soil infusions may not be absolutely representative, and hence the results secured by the method cannot be depended upon as representing field conditions.

In short, the casein method according to these results, possesses many advantages over the other methods and previous claims made for it are entirely supported.

THE NITRIFICATION EXPERIMENTS.

The nitrification experiments were carried out by using ammonium sulfate with fresh soil and with air-dry soil. The use of fresh soil has seemed by far the most rational method in examining soils, and it was desired to compare its results with those obtained with air-dry soil.

One hundred gram quantities of fresh soils were weighed off *in tumblers, stirred thoroughly and one* c.c. *portions of a 10%* solution of ammonium sulfate added.

The moisture content of the soils was ascertained and then brought up to the optimum with sterile water. The tumblers were covered and incubated for four weeks, the water content being kept constant by making up to weight with sterile water every week.

At the same time 100-gram quantities of air-dry sieved soil from the various plots were weighed off, one c.c. portions of a 10% ammonium sulfate solution added, and twenty c.c. of five minute infusions of fresh samples of the corresponding soils

TABLE XII. THE NITRIFICATION OF $(NH_4)_2 SO_4$.
(Fresh Soil)

Plot No.	Lab. No.	I Aug. 2 Mgs. N.	Average Mgs. N.	Lab. No.	II Aug. 15 Mgs. N.	Average Mgs. N.
1004	141	5.000		641	11.000	
	142	6.152	5.576	642	10.892	10.946
1005	143	7.156		643	12.500	
	144	7.362	7.259	644	12.667	12.583
1006	145	8.379		645	16.665	
	146	8.561	8.470	646	16.802	16.733
1007	147	10.164		647	18.500	
	148	10.400	10.282	648	18.888	18.694
1008	149	8.052		649	16.000	
	150	8.199	8.125	650	16.328	16.164

Plot No.	Lab. No.	III Aug. 22 Mgs. N.	Average Mgs. N.	Lab. No.	IV Sept. 9 Mgs. N.	Average Mgs. N.
1004	1141	10.000		1341	9.282	
	1142	10.567	10.283	1342	9.000	9.141
1005	1143	12.339		1343	10.000	
	1144	12.738	12.543	1344	10.000	10.000
1006	1145	14.285		1345	12.500	
	1146	14.000	14.142	1346	12.896	12.698
1007	1147	15.282		1347	12.800	
	1148	16.000	15.641	1348	13.222	13.011
1008	1149	13.009		1349	10.228	
	1150	12.809	12.949	1350	10.829	10.528

TABLE XIII. THE NITRIFICATION OF $(NH_4)_2SO_4$.
(Fresh Soil)

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	5.576	10.946	10.283	9.141
1005	7.259	12.582	12.543	10.000
1006	8.470	16.733	14.142	12.698
1007	10.282	18.694	15.641	13.011
1008	8.125	16.164	12.949	10.528

were introduced. The moisture content was adjusted and maintained during the incubation period of four weeks as in the other method.

The results of the tests with fresh soils are given in table XII. The duplicate determinations agreed very satisfactorily as shown in table XIII which gives the summarized results.

In every case the manure increased the nitrifying power of the soil up to sixteen tons per acre. Beyond that, however, a reduction occurred, twenty tons depressing the nitrate production below that shown by the soil receiving twelve tons per acre. The results were quite in agreement with those secured in the quantitative determinations and ammonification experiments except that in those cases the maximum amount of manure was not so definitely shown. Here the greatest nitrifying power in every case appeared in the soil receiving sixteen tons of manure per acre.

The results by the use of air-dry soil are given in table XIV and here, too, the duplicates were quite satisfactory. In table XV the summarized results of the tests appear and upon examination it is found that differences in the nitrifying power of the soil were obtained here similar to those secured by the use of fresh soil. Again the application of manure up to sixteen tons per acre increased the nitrifying power of the soils, twelve tons giving a larger nitrate production than eight tons, and sixteen tons more than twelve. There was one exception to this increase, however, at the last date of sampling a slightly smaller nitrate production was noted in the plot receiving sixteen tons of manure than in that to which twelve tons were applied. The difference was so slight that it would be negligible were it not for the fact that the same slight decrease occurred at this date in the numbers of organisms and in the ammonifying power measured by casein or by dried blood in fresh soil, in the soil receiving sixteen tons over that to which twelve tons per acre were added. It might seem therefore that at that particular date, or in the particular sample some peculiarity or unavoidable contamination altered the results from their common trend at the other dates of sampling.

Here again the application of twenty tons of manure depressed the nitrifying power of the soil below that shown when only twelve tons were applied.

The results also check quite satisfactorily the results of the quantitative determinations and the ammonification experiments.

As a whole the nitrification experiments bring out the same facts as were obtained by the ammonification and quantitative studies. The increases in numbers and ammonifying power were accompanied by increases in nitrifying power. Depressions in numbers and ammonia production were likewise fol-

TABLE XIV. THE NITRIFICATION OF $(NH_4)_2SO_4$.
(Air-dry Soil)

Plot No.	Lab. No.	I Nitrate Mgs. N.	Average Mgs. N.	Lab. No.	II Nitrate Mgs. N.	Average Mgs. N.
1001	121	8.335		621	14.689	
	122	8.679	8.507	622	14.900	14.794
1005	123	9.260		623	15.350	
	124	9.392	9.326	624	15.557	15.453
1006	125	10.000		625	17.892	
	126	10.000	10.000	626	17.528	17.710
1007	127	11.905		627	18.500	
	128	11.405	11.655	628	18.925	18.712
1008	129	10.000		629	16.667	
	130	10.128	10.064	630	16.725	16.696

Plot No.	Lab. No.	III Nitrate Mgs. N.	Average Mgs. N.	Lab. No.	IV Nitrate Mgs. N.	Average Mgs. N.
1004	1121	12.500		1221	9.422	
	1122	12.500	12.500	1222	9.000	9.211
1005	1123	13.800		1223	10.000	
	1124	13.586	13.693	1224	10.525	10.262
1006	1125	14.285		1225	12.500	
	1126	14.500	14.392	1226	12.687	12.593
1007	1127	16.666		1227	12.000	
	1128	16.137	16.401	1228	12.892	12.446
1008	1129	14.500		1229	10.000	
	1130	14.825	14.662	1230	10.889	10.444

TABLE XV. THE NITRIFICATION OF $(NH_4)_2SO_4$.
(Air-dry Soil)

Plot No.	I Nitrate Mgs. N.	II Nitrate Mgs. N.	III Nitrate Mgs. N.	IV Nitrate Mgs. N.
1004	8.507	14.794	12.500	9.211
1005	9.326	15.453	13.693	10.262
1006	10.000	17.710	14.392	12.593
1007	11.655	18.712	16.401	12.446
1008	10.064	16.696	14.662	10.444

lowed by decreases in nitrate production. The amount of manure bringing about maximum bacterial development among ammonifying species also caused maximum multiplication and activity of the nitrifying species. Previous results which showed that ammonification and nitrification proceeded parallel were confirmed by the work at hand.

The reasons for the increases and decreases in ammonifying and nitrifying power by the use of manure may be assumed to be the same as were mentioned in the discussion of the quantitative results. That is, the increases may be due either to additions of ammonifying and nitrifying organisms, or to the abundant bacterial food supply added which encourages those organisms already present in the soil to greater development. The decrease when too much manure was used may be due to action of the organic matter in the manure on the ammonifying and nitrifying organisms or possibly to the introduction of competing organisms to such an extent that the prominent ammonifiers and the nitrifiers were hindered in their development.

Comparing the nitrifying power of the soils as shown by the use of fresh and air-dry soil, much greater differences were found between the different soils when fresh soil was used. The greater differences are apparently due to the fact that the nitrifying power of the check soil was lower when tested by the fresh soil than when air-dry soil was used. Just why this should be so is not apparent, but there can be no doubt but that the fresh soil approaches more closely the natural conditions and hence is more nearly representative of what is occurring in the field.

THE CROP YIELDS.

Table XVI gives the yields of corn for 1912 from the plots used in these experiments. The figures given show that eight tons of manure increased the yield from 50.50 bushels to 77.62 bushels; twelve tons increased this to 86.00 bushels and sixteen tons gave a slight gain to 87.00 bushels. Twenty tons, however, caused a depression in yield to 81.00 bushels, which was below that from the plot receiving twelve tons.

Comparing these results with the bacteriological data almost absolute agreement is found. Eight tons of manure increased the number of organisms, it increased the ammonifying and nitrifying power of the soils over the check soil and likewise increased the crop yield. Twelve tons gave a further increase in crop, corresponding to an increase in numbers of bacteria, and in ammonifying and nitrifying powers. Sixteen tons showed a very slight gain in crop, and with a few exceptions, where the

TABLE XVI. THE CROP YIELDS.
(Treatment and Yield per acre.)

Plot No.	Treatment	Corn (1912)
1004	Check	50.50 bu.
1005	8 T. Manure	77.62 bu.
1006	12 T. Manure	86.00 bu.
1007	16 T. Manure	87.00 bu.
1008	20 T. Manure	81.00 bu.

differences were very small, a slight gain in numbers of bacteria and in ammonifying and nitrifying power occurred. Finally twenty tons of manure per acre depressed the crop yield below that produced with twelve tons and the numbers of bacteria, ammonifying and nitrifying powers of the soil were depressed to just that extent.

These results therefore give additional confirmation to the conclusion drawn in previous study of field soils that bacterial activities and crop production were very closely related. Furthermore, the suggestion that the determination of bacterial activities may be a means of determining the fertility or crop-producing power of a soil or at least the relative fertility of several soils is worthy of consideration in the light of the present results.

It may be suggested that the depression in crop yields by the application of twenty tons of manure per acre is due to denitrification and that the decrease in numbers, of bacteria, etc., is due to the introduction of denitrifying bacteria. This theory, however, will not account for the results secured, as tests of the denitrifying power of the soils were carried out both by the use of the Giltay solution and by the use of soil cultures, and in no case was any denitrification observed. The nitrates used in the media very rapidly disappeared but upon analysis the entire amount has been found to have been changed into protein, and no loss whatever occurred.

It may be stated here that it is probably the case that many instances of reported denitrification have been made on the basis of the disappearance of nitrates, and if chemical analyses had been made no losses of nitrogen would have been found.

At any rate the depression in crop yield which followed application of a large amount of manure cannot be attributed to denitrification in this case but to some other influence, possibly as has been suggested, physiological, and the danger of denitrification in field soils which has been emphasized in some quarters may be regarded as open to question.

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THE DETERMINATION OF AMMONIA IN SOILS

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THE DETERMINATION OF AMMONIA IN SOILS

By R. S. Potter and R. S. Snyder

INTRODUCTION.

Theoretically, the ideal method for determining ammonia in soils would give the absolute amount of ammonia present as such and as the ammonium radical, but in the light of present knowledge, it cannot be said beyond all doubt that any conceivable method would give this ideal result. This is true because a large part of the nitrogen of the soil is present in protein and protein degradation products, the extensive decomposition of which gives large amounts of ammonia. The uncertainty regarding just what products are present does not permit the finding of conditions which we can be certain will expel ammonia already present and not decompose any material to give, among other products, ammonia. The problem is further complicated by the well known absorptive and adsorptive powers of the soil.

REQUIREMENTS OF A METHOD FOR AMMONIA.

Therefore, since this ideal result is not attainable, in our opinion the value of any method will depend upon whether it fulfills the following requirements:

1st. Closely agreeing duplicate results should be given and the same result obtained whether the reagent or reagents act, within reasonable limits, for a longer or shorter period. Of course, the reagent or reagents must not include any which are known to decompose material contained in the soil to give ammonia. For instance, it would not be permissible to use strong hot hydrochloric acid, for this reagent decomposes proteins yielding large amounts of ammonia.

2nd. Upon the addition of a known amount of ammonia, the method must give this added amount plus that previously found in the soil. Not enough time, of course, should elapse between the addition of the ammonia and the determination for any bacterial action to take place. In soils already containing ammonia and, apparently all soils do, it is difficult to see how any chemical reaction could, in a reasonably short length of time, change the added nitrogen to some other combination than the ammonium. Because his method does not recover all added ammonia, Russel assumes the ammonia to be chemicaly changed. This will be further discussed later.

3rd. For use in a soils laboratory, the method should

permit one to run several determinations within a reasonable length of time.

METHODS WHICH HAVE BEEN PROPOSED.

The folloing methods have been proposed:

I. Schloesing's Method[1] "A"
II. Schloesing's Method[1] "B"
III. Boussingault's Method[2]
IV. Wolf & Knop's Method[3]
V. Baumann's Method[4]
VI. Russel's Method[5] "A"
VII. Russel's Method[5] "B"

I. Schloesing's first method consists in leaving a mixture of the soil with strong sodium hydroxide solution under a bell jar, together with a vessel containing some standard acid to absorb the ammonia evolved. This method has fallen into complete disuse because of the recognition of the fact that strong sodium hydroxide solution, even in the cold, would graduall decompose the organic nitrogen compounds to give ammonia.

II. In Schloesing's second method, the soil is treated with dilute hydrochloric acid until the liquid remains distinctly acid. The mixture is then well shaken, filtered and an aliquot portion is distilled with alkali. Since this method complies with the third requirement named above, and since we can find no evidences in the literature that it does or does not comply with the first two, we have given the method a rather detailed examination, the results of which are reported and discussed in the experimental part of this paper.

III. In Boussingault's method a mixture of about one part of soil to two parts of water, together with a little magnesia, is distilled into standard acid. It is quite generally admitted that this method does not give true results with material like soil which contains protein and protein degradation products. Yet, because of its ease and simplicity of manipulation, it is often used, it being stated that the results, although not absolute, are comparable with one another. This method has also been examined by us and the results will be taken up in the experimental part of this paper.

IV. The method of Wolf & Knop consists in treating the soil with sodium hypobromite, which reacts with the ammonia, liberating nitrogen gas, which is measured. Baumann[4] has shown this method does not give accurate results.

V. In Baumann's method a hydrochloric acid extract is made as in Schloesing's second method. To this extract, magnesia is added and ozone is bubbled through the solu-

1. Analyse des Matieres Agricoles. 1879.
2. Agronomie 1864: 206.
3. Chem. Centralb. 1860: 243, 253.
4. Landw. Vers. Sta. 33; 247. 1886
5. Jour. of Ag. Sci. 1910: 233.

tion, and it is then treated with sodium hypobromite and the nitrogen collected and measured. In view of the facts which are brought out below in regard to the hydrochloric acid extract of soils, together with the fact that the sodium hypobromite used contained an excess of sodium hydroxide, which would gradually decompose nitrogenous organic compounds to give ammonia, methods IV and V may be considered as unreliable.

VI. In Russel's first method, 150 grams of soil are distilled at the pressure obtained with a water pump with 2 grams of magnesia suspended in 100 c.c. of water. The distillation ask is kept at 40° C., and the distillation is continued for six hours.

VII. Russel's second method is similar to his first method, except that the 2 grams of magnesia are replaced by 0.7 gram potassium magnesia hydroxide and the 100 c.c. of water by an equal volume of alcohol, and the temperature of the distillation ask is kept at 25° C. instead of 40° C. Russel states that insofar as he has tested his methods, they give practically identical results. He also points out that with the use of magnesia there is some decomposition. Indeed, with the soil upon which he reports results he obtained both on the second and third distillation as much ammonia as was obtained on the first.

Although Russel shows that his potash method gives concordant results and no ammonia is given off after a reasonable length of time (first requirement), yet upon addition of ammonia to the soil, only from 50 to 84 per cent is recovered by either method. Hence, they do not comply with the second requirement. He explains this by stating that the ammonia enters into a stable combination which is not an ammonium compound. It could be argued with equal force that the ammonia forms a relatively insoluble ammonium compound with the complex organic or inorganic material of the soil, or that it was physically absorbed. It does not seem surprising that all the ammonia was not expelled when there was 50 c.c. of water left with the soil after the distillation was stopped, and the high solubility of ammonia[1] and the low solubility of magnesium hydroxide[2] is considered.

EXPERIMENTAL.

In the following work, the ammonia free water which was used was prepared by boiling ordinary tap water for a

1. Seidell, Solubilities of Inorganic and Organic Substances, 1911: 17. 30.7 grams per 100 grams water at 40° C. and 760 mm. pressure. At the pressure Russel used, the ammonia would be soluble to the extent of approximately one gram per 100 grams water.
2. Ibid.: 1. 181. 0.008-0.009 grams $Mg(OH)_2$ per liter of water at 18° C.

few minutes with about two grams of potassium acid sulphate to one liter of water, and then distilling the solution and condensing the vapor in block tin tubes which had previously been steamed for 12 hours. The distillate in every case was found to be neutral to alizarin red and gave but a slight indication of ammonia when tested with Nessler's solution.

Whenever magnesia was used, it was always freshly calcined.

Alizarin red was the indicator used in all ammonia titrations. This has been found to be by far the most satisfactory indicator for ammonia titrations, and is now exclusively used for that purpose in this laboratory.

Blanks were always run and the suitable corrections made. Duplicates were not run, except as reported.

The following soils were used in this work:

Soil No. 1—Southern Iowa loess.
Soil No. 2—Iowa drift.
Soil No. 3—Wisconsin drift.
Soil No. 4—Mississipi lopess.
Soil No. 5—Missouri loess.
Soil No. 6—Alkali I.
Soil No. 7—Alkali II.

Soils nos. 1 to 5 were typical samples from the five principal soil areas of Iowa. Soils nos. 6 and 7 were from the Wisconsin drift area.

AMMONIA IN THE HYDROCHLORIC ACID EXTRACT OF SOILS.

To find out whether the Schloesing method would give reliable results, it was examined as follows:

1. The strength of the hydrochloric acid used for extraction was varied.
2. The time of extraction was varied.
3. Known amounts of ammonia as sulphate were added to the soil and it was then extracted with different strengths of hydrochloric acid for different lengths of time.

All of the acid extracts were obtained by shaking in a mechanical shaking machine for the indicated length of time a mixture of one part of air dry soil with two parts of ammonia free acid. When ammonia was added, it was introduced as standard ammonium sulphate solution immediately before the addition of acid of suitable strength to provide the desired normality. Thus, any appreciable bacterial change of the ammonia was precluded. After removing from the shaking machine, the mixture was filtered through a double folded filter, a clear filtrate being obtained in all cases. For the magnesia distillation, 200 c.c. of the filtrate, repre-

senting 100 grame of soil, were distilled after the addition of about 10 grams of magnesia until 150 c.c. of distillate had been collected. In some cases 0.1 N acid and alkali were used in the titrations, and in others 0.02 N. The results of the magnesia distillations are given in table I.

TABLE I.

Soil No.	Lab. No.	Strength of Acid	Time Shaken, in Min.	Ammonia Added in Mgs. N	Ammonia Found in Mgs. N	Average in Mgs. N	Recovered in Mgs. N	Per Cent Recovered
1	1	0.2N	20 min.		1.26			
1	1	0.2N	"		1.19	1.23		
1	3	0.3N	"		1.26			
1	4	0.3N	"		1.26	1.26		
1	5	0.2N	60 min.		1.26			
1	6	0.2N	"		1.23	1.25		
1	7	0.3N	"		1.26			
1	8	0.3N	"		1.26	1.26		
1	9	0.2N	36 min.	2.25	2.60			
1	10	0.2N	"	2.25	2.60	2.60	1.35*	60.0
1	11	0.3N	"	2.25	2.60			
1	12	0.3N	"	2.25	2.64	2.62	1.37*	60.9
3	13	0.2N	20 min.		0.772			
3	14	0.3N	"		0.842			
3	15	0.2N	60 min.		0.814			
3	16	0.3N	"		0.842			
3	17	0.2N	20 min.	2.25	2.32		1.55	68.0
3	18	0.3N	"	2.25			1.38	61.3
3	19	0.2N	60 min.	2.25	2.25		1.44	64.0
3	20	0.3N	"	2.25	2.36		1.32	67.6

*In computing these values the average of the averages of nos. 1 to 8 were used

The results in table I show that within reasonable limits no ammonia enters into solution after 20 minutes, and that also within the limits of the experiment the amount of ammonia dissolved is independent of the strength of acid. It is also shown that not nearly all the ammonia which was added was recovered. The rather wide variance of the percentages recovered in the case of soil no. 3 is due to the error in the determination of so small an amount of ammonia as was in this soil.

Since the hydrochloric acid extract undoubtedly contains some organic nitrogenous material, it was of some interest to subject the residues in several of the distillation flasks to a second distillation. Accordingly, 150 c.c. of ammonia free water was added to each of the flasks of nos. 1 to 4 and 150 c.c. distilled as before. The results are given in table II.

It is seen that an appreciable amount of ammonia is given in each instance, and this no doubt comes from a decomposition of organic material. To further test this point

TABLE II.

Lab. No.	Residues from No.	Ammonia Found, in Mgs. N
21	1	0.28
22	2	0.27
23	3	0.42
24	4	0.39

and to test the applicability of Folin's[1] method for ammonia to the hydrochloric acid extract of soils, 100 c.c. portions of the same filtrates of which 200 c.c. were used in experiments nos. 1 to 4 were aerated for four hours, after the addition of 4 grams of sodium carbonate, in an apparatus which will be described later. The results are set forth in table III, and are for 100 grams of soil. For purposes of comparison, the results obtained by distillation with magnesia are repeated here.

TABLE III.

Lab. No.	From same Filtrate as was used in Nos.	Ammonia found by Aeration, in Mgs. N	Ammound found by Distillation, in Mgs. N
25	1 and 2	1.26	1.23
26	3 and 4	1.28	1.26
27	5 and 6	1.26	1.25
28	7 and 8	1.29	1.26

The aeration was continued for one more hour, but no ammonia was given. The results obtained by the aeration method do not vary from those found by distillation by more than the experimental error. Owing to the possibility of decomposition with magnesia at 100° C. one would expect the results obtained by aeration to be slightly lower. The results show that this method can be applied to the hydrochloric acid extract and its use in preference to the distillation method is recommended, both from the standpoint of accuracy and ease of manipulation.

In table IV are given the results obtained by aerating the acid filtrates from soils nos. 2, 4 and 5 as outlined above. For purposes of comparison, the results are calculated for 100 grams of soil, although as above they were obtained on 100 c.c. of filtrate, representing 50 grams of soil.

The results recorded in table IV bring out about the same points as those in table I, namely, that the amount of ammonia extracted is, within the limits of the experiment, independent of the strength of acid or the time of extraction, and that not all of the added ammonia is recovered. For the soils tested, and these represented quite a range of soil types, we are forced to the conclusion that any method which

1. Zeit. f. physiol. Chem. 37: 161. 1902.

TABLE IV.

Soil No.	Lab. No.	Strength of Acid	Time Shaken in Min.	Ammonia Added, N in Mgs.	Ammonia found, N in Mgs.	Ammonia recovered in Mgs. N	Per Cent recovered
5	29	0.2N	20 min.		1.10*		
5	30	0.3N	"		1.21		
5	31	0.2N	60 min.		1.24		
5	32	0.3N	"		1.24		
5	33	0.2N	20 min.	2.25	2.63	1.40	62.2†
5	34	0.3N	"	2.25	2.63	1.40	62.2†
5	35	0.2N	60 min.	2.25	2.59	1.36	60.4†
5	36	0.3N	"	2.25	2.63	1.40	62.2†
2	37	0.2N	30 min.		1.24		
2	38	0.2N	"	2.25	2.80	1.56	69.3
4	39	0.2N	"		0.53		
4	40	0.2N	"	2.25	1.93	1.40	62.2

*Probably an error.
†In computing these results, the average of the results obtained in experiments 30, 31 and 32 was used.

requires an examination of the hydrochloric acid extract is not reliable. It might indeed be contended that treatment with fresh portions of hydrochloric acid would finally recover all the added ammonia. It is questionable whether this point could easily be proven, for no doubt long continued treatment of the soil even with acid of this dilution would gradually decompose organic matter with the production of amomnia. It was not thought worth while to investigate this point, for even if a method could be elaborated, it probably would not comply with the third requirement, namely that of practicability. As to the cause of the failure to recover all the added ammonia, any explanation would be purely speculative. In the course of some work which we shall publish later, we have found that the use of too rigorous flocculating agents to clear the solution for the determination of nitrates in the soil causes a decided lowering in the amounts of nitrates found. It is possible that the phenomenon of incomplete recovery of ammonia is due to the flocculating action of the acid.

DISTILLATION OF SOIL AT ORDINARY PRESSURE WITH MAGNESIA.

It has been shown[1] that successive distillation with magnesia of such substances as beef, eggs, dried blood and cottonseed meal gives for several distillations small but appreciable amounts of ammonia, but so far as we know, this method has never been critically examined with regard to

1. Trescott, Bul. U. S. Dept. of Ag., Bur. of Chem., 132: 20.

soil. We have accordingly carried out the following tests:

One hundred grams of soil were placed in a copper flask, together with 200 c.c. of ammonia free water, a small piece of paraffin and about 100 grams of magnesia and distilled until 150 c.c. of distillate had been collected in standard acid. The receiving flask was removed and replaced by another with a suitable quantity of standard acid. One hundred and fifty c.c. of ammonia free water were then put in the copper flask and 150 c.c. of distillate were again collected. This was repeated a third, and in the case of one soil, a fourth time. The results are given in table V.

TABLE V.

Soil No.	Lab. No.	Ammonia, 1st distillation, in Mgs. N	Ammonia, 2nd distillation, in Mgs. N	Ammonia, 3d distillation, in Mgs. N	Ammonia, 4th distillation, in Mgs. N
1	41	3.51	1.54	.91	
1	42	3.16	1.54	.91	
2	43	3.70	1.26	.84	
2	44	3.80	1.26	.84	
3	45	2.87	1.12	.81	
3	46	2.67	1.12	.84	
4	47	2.46	.98	.70	.70
4	48	2.53	.84	.70	.63
5	49	3.16	.77	.98	
5	50	3.26	.77	.91	

From the results recorded in table V, it is apparent that the amount of ammonia obtained by distillation is dependent upon the durtaion of the distillation, which is, of course, proportionate to the amount of water and soil used and upon the quantity of heat applied. The results of the following experiments emphasize the latter point in a striking way. For each experiemnt, 100 grams of soil, 200 c.c. of ammonia free water, a small piece of paraffin and 10 grams of magnesia were used. The full flame of a large burner was used on each of the flasks of nos. 51, 52, 55 and 56. The flames played directly upon the flasks, while in nos. 53, 54, 57 and 58, a low flame was used, and each flask was protected with a wire gauze. The results are given in table VI.

From a consideration of the results in table VI, although the conditions have been made extreme, there seems to be room for doubt as to whether even comparable results can be obtained by distillation with magnesia. Although one might regulate the gas flame during the distillation of a single series so that each gave about the same heat, yet the results obtained on different days or at a different time on the same day might be appreciably variable.

TABLE VI.

Soil No.	Lab. No.	Time in minutes of distillation	Mgs. N. as Ammonia
1	51	40	2.80
1	52	40	2.67
1	53	150	4.63
1	54	150	4.63
2	55	45	3.37
2	56	40	3.09
2	57	140	4.49
2	58	160	5.34

It is obvious, therefore, from the data presented above and from the work of others previously cited, that there is no very reliable method in use for the determination of ammonia in soils. That it is highly desirable to have such a method is apparent from the general importance of the soil nitrogen problem, particularly in the study[1] of the influence of lime and various other substances added to soil upon its ammonia content; in the study of the ammonia exaporation from soils; in the study[2] of the direct assimilation of ammonia by higher plants, and in the study of the ammonia problem in many other practical and theoretical phases. The attempt was made, therefore, to find a method for the determintaion of ammonia in the soil which would comply with the three requirements named in the introduction to this paper. The results as given below will show to what extent the attempt has succeeded.

AMMONIA IN THE SOIL BY AERATION.

In 1902, Folin[3] outlined the method which, with some modifications, is largely used for the determination of ammonia in urine and various other physiological products. Originally the method involved drawing air at the rate of 600 to 700 liters per hour for 1 to 1½ hours through 25 c.c. of urine containing 1 gram of sodium carbonate and 8 to 10 grams of sodium chloride. From the cylinder containing the urine the air passed through standard acid, which absorbed the ammonia. Steel and Gies[4] found that with urines containing magnesium ammonium phosphate crystals, low results were given because this salt was but slowly decomposed by sodium carbonate. It was later shown[5] that all urines,

1. Ehrenburg. Landw. Ztg. **60:** 441, 479, 1911. Von Klodeck. Jour. Chem. Soc. (Eng.) II **102:** 85; O. Lemmermann and L. Fresenius. Landw. Jahrb. **45:** 127. 1913.
2. Hutchinson & Miller. Jour. Ag. Sci. III. 179.
3. Zeit. fur physiol. Chem. **37:** 161. 1902.
4. Jour. Biol. Chem. 5: 71. 1909.
5. Benedict & Osterburg. Biecham. Bul. III, 41. 1913.

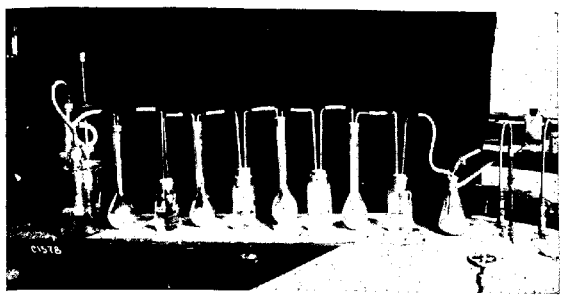
upon the addition of sodium carbonate, gave copious deposits of magnesium ammoniu mphosphate and, consequently, low results for ammonia. Steel[1], therefore, recommends the use of 0.5 gram sodium hydroxide in place of the 1 gram of sodium carbonate. He found that with the use of these reagents (sodium hydroxide and sodium chloride) all the ammonia was readily given off from triple phosphate crystals, and that apparently they did not decompose any organic materials, as no ammonia was given after four hours' aeration. He also found that aeration of solutions of each of the following substances gave no ammonia: Glycine, urea, uric acid, leucine, tyrosine, hippuric acid, guanine, allantoin, creatine and creatinine. Of these compounds, creatinine[2] and guanine[3] have been isolated from the soil by such methods as would make it appear that they were present as such and not in combination. Tyrosine[4] and leucine[5] have been isolated from the solution obtained by long continued boiling of the soil with hydrochloric acid.

Since the aeration methods have been so successfully used in connection with ammonia determinations in urine, which contains a wide variety of organic nitrogenous products, we decided to make an attempt to modify it for use with soil. Since ammonia has been shown by Russel[6] and the authors of this bulletin to be rather tenaciously held by the soil, the stronger reagent used in Steel's modification was first tried. In the course of some preliminary investigations, various forms of aeration aparatus were tried, but the form which was found most satisfactory was similar to the apparatus used by Kober[7] in his "Ammonia Distillation by Aeration" method. We use a 16 oz. bottle for the absorption bottle, and a 500 c.c. round bottom Kjeldahl flask for the aeration flask. The air and ammonia enter the absorption bottle through a specially made absorption tube. The directions for making this tube are given by Folin[8].

For any further details as to the setting up of the apparatus, the accompanying illustration should be consulted.

The filtration flask must always be set perpendicular to the table, and the long tube in the aeration flask should reach to within not more than 3 millimeters of the bottom of the flask. Both of these last precautions are necessary to secure adequate stirring of the mixture. Only 4 units are

1. Jour. Biol. Chem. 8: 365. 1910.
2. Sharey. Bul. U. S. D. of Ag. Bur. of Soils 83. 1911.
3. Lathrop. Jour. Am. Chem. Soc. 34: 1260. 1912.
4. Suzuki. Bul. Coll. Tokyo 7: 513.
5. Robinson. Tech. Bul. Mich. Ag. Exp. Sta. 7.
6. Loc. Cit.
7. Jour. Am. Chem. Soc. 35: 1594. 1913.
8. Loc. Cit.



The Aeration Apparatus

shown in the cut, but as many as 14 determinations in series on one pump have been run. For all the work reported in this paper, a current of air of about 250 liters per hour was used.

In our trial of the Steel method, the following technique was used: The "alkali" was prepared by saturating ammonia free water wtih sodium chloride. In order to be always sure that a saturated solution was obtained, the calculated amount of the salt was added to a known volume of water. Sodium hydroxide was added at the rate of 2 grams per 100 c.c. of water. Twenty-five grams of soil were placed in a Kjeldahl flask. The absorption bottle was half filled with ammonia free water[1] and 100 c.c. of 0.02 N. sulphuric acid added. The apparatus was then connected up, the stopper replaced and the flask shaken and the suction started.

The results are set forth in table VII, and are computed to 100 grams of air dry soil. For purposes of comparison, the results obtained for the corresponding soils on the first distillation with magnesia are included.

Since after aerating 15 hours ammonia was being expelled in relatively large amounts, and because the amounts given off are far in excess of those given off by distillation with magnesia, there is no other conclusion but that there is a decided decomposition of organic nitrogenous matter to give ammonia. The work of Steel[2] shows that probably this excess ammonia is not due to a decomposition of amino acids. Another class of nitrogenous compounds less stable

1. The reason for using ammonia free water here was that a sharper end point was obtained with it than when ordinary distilled water was used in the nitration. This was, no doubt, due to a slight trace of amphoteric matter in the distilled water.
2. Loc. Cit.

TABLE VII.

Soil No.	Lab. No.	Time of aeration in hours	Ammonia in Mgs. N	Ammonia by MgO, in Mgs. N
1	59	4½	3.87	
1	60	4½	3.93	
1	61	6	4.49	
1	62	6	4.63	
1	63	8	5.61	
1	64	8	5.56	
1	65	15	7.02	
1	66	15	7.30	
1	67	19	8.70	
1	68	19	8.99	3.33
3	69	15	4.21	
3	70	15	4.49	
3	71	19	6.60	
3	72	19	6.46	2.67
5	73	15	7.58	
5	74	15	7.86	
5	75	19	9.13	3.21
5	76	19	9.41	

in the presence of alkalies than the amino acids is the acid amides. Kelly and Thompson[1] in analyses of the alkali extract of nine soils, found an average of 14.3 per cent amide nitrogen. Jodidi[2], Robinson[3] and others have found from 20 to 30 per cent of amide nitrogen in the solution obtained by boiling the soil for several hours with strong acid. But because of their inherent instability, it seems highly improbable that acid amides are present in soils in much larger, if as large, percentages as ammonia. Many acid amides would slowly decompose in the presence of the Steel reagent. For instance, we found that 0.1 gram acetamide, purified by recrystallization from ether, in two hours aeration with the Steel reagent, gave 1.54 mgs. ammonia and in four hours, 2.56 mgs. It seem squite probable, therefore, that at least part of the excess ammonia is due to the decomposition of acid amides.

It was next decided to try the use of sodium carbonate as the alkali. Accordingly, as a preliminary test, 0.1 gram of acetamide was dissolved in 50 c.c. of water, 2 grams of sodium carbonate added and the solution aerate. At the end of two hours, 0.08 mg., and at the end of twenty hours 0.02 mg. of ammonia was given. No doubt the production of even the 0.08 mg. during the first two hours was from a trace of ammonium salts in the acetamide.

1. Jour. Am. Chem. Soc. **36:** 438. 1914.
2. Tech. Bul. Mich. Ag. Exp. Sta. 4. 1909. Research Bul. Iowa Ag. Exp. Sta. 1 and 3. 1911.
3. Loc. Cit.

As is stated above, it has been found that all urines upon the addition of sodium carbonate give triple phosphate crystals, and on aeration low results are given for ammonia. Since magnesium, ammonium and phosphate are present in the soil solution, it would seem that the same thing would take place as with urines, namely, low results with sodium carbonate. In 1908 Folin[1] found that this objection in the case of urine could be overcome by the addition of potassium oxalate to the solution before the addition of sodium carbonate. If the magnesium ammonium phosphate was already there as a precipitate, the solution should first be made acid, which would dissolve the crystals and then the potassium oxalate added. This same procedure could, of course, be applied directly to any solution besides urine. We have, therefore, tried the use of sodium carbonate with and without the addition of potassium oxalate on seven soils, and in the case of one soil, we have tried sodium chloride and sodium carbonate. The results are all grouped together in table VIII, and are for 100 grams of soil.

In all cases, 25 grams of soil, 50 c.c. of ammonia free water and about 2 grams of sodium carbonate, and where indicated 8 to 10 grams of potassium oxalate and 15 to 17 grams of sodium chloride, were used. Also, when potassium oxalate was used, the mixture was always made slightly acid before the addition of the sodium carbonate.

An examination of the foregoing table brings out many interesting points. For the soils tested, sodium carbonate used alone causes no appreciable decomposition of organic matter. It is possible that with a soil high in organic matter, it might be advantageous to use sodium chloride, which, of course, acts to weaken the base. The use of potassium oxalate, it is seen, gives no more ammonia than sodium carbonate alone. This is at first thought somewhat surprising, but no doubt the reason for this is that the phosphate and ammonia are present in such high dilution in the soil solution that the magnesium ammonium phosphate does not precipitate. In urine[2] the phosphate content averages about 2000 parts per million, and the ammonia[3] 1000 parts per million, while in the soil King[4] found as a maximum amount of water soluble phosphate about 20 parts per million, and we have found as a maximum amount of ammonia about 30 parts per million. Since by our method 2 parts of water to 1 part of soil are used, these values should be halved. It is,

1. Jour. Biol. Chem. **8**: 497. 1910.
2. Hammarsten. Text Book of Physiological Chemistry. 646. (6th Eng. ed.)
3. Hawk's Practical Physiological Chemistry. 313. (4th ed.)
4. King. Bul. U. S. Dept. of Ag., Bur. of Soils. 26.

TABLE VIII.

Soil No	Lab. No.	Time of aeration in hours	Alkali reagent	Ammonia added, in Mgs. N	Ammonia found in Mgs. N	Average, in Mgs. N	Ammonia recovered, in Mgs. N	Per Cent recovered
1	78	15	Na^2CO^3, NaCl		2.08			
1	79	15	" "		2.08	2.08		
1	80	15	" "	2.25	4.29			
1	81	15	" "	2.25	lost	4.29	2.21	98.2
1	82	19	" "		1.97			
1	83	19	" "		2.08	2.03		
1	84	19	" "	2.25	4.29			
1	85	19	" "	2.25	4.27	4.28	2.25	100
1	86	19	Na_2CO_3		2.08			
1	87	19	"		1.97	2.03		
1	88	19	"	2.25	4.27			
1	89	19	"	2.25	4.32	4.30	2.27	100.9
1	90	15	$K_2C_2O_4$, Na_2CO_3		2.08			
1	91	15	" "		2.08	2.08		
1	92	15	" "	2.25	4.32			
1	93	15	" "	2.25	4.27	4.30	2.22	98.7
1	94	19	" "	2.25	4.32			
1	95	19	" "	2.25	4.32	4.32	2.24	99.5
2	96	15	Na_2CO_3		2.59			
2	97	15	"		2.53	2.56		
2	100	15	"	2.25	4.84			
2	101	15	"	2.25	4.78	4.81	2.25	100
2	102	19	"		2.53			
2	103	19	"		2.55	2.54		
2	104	19	"	2.25	4.60			
2	105	19	"	2.25	4.78	4.78	2.24	99.5
2	106	15	$K_2C_2O_4$, Na_2CO_3		2.59			
2	107	15	" "		2.59	2.59		
2	108	15	" "	2.25	4.84			
2	109	15	" "	2.25	4.78	4.81	2.22	98.7
2	110	19	" "	2.25	4.71			
2	111	19	" "	2.25	4.84	4.78	2.19	97.3
3	112	15	Na_2CO_3		1.23			
3	113	15	"		1.23	1.23		
3	114	15	"	2.25	3.48			
3	115	15	"	2.25	3.54	3.51	2.28	101.3
3	116	19	"		1.23			
3	117	19	"		1.18	1.21		
3	118	19	"	2.25	3.48			
3	119	19	"	2.25	3.48	3.48	2.27	100.9
3	120	15	$K_2C_2O_4$, Na_2CO_3		1.23			
3	121	15	" "		1.18	1.21		
3	122	15	" "	2.25	3.48			
3	123	15	" "	2.25	3.54	3.51	2.30	102.1
3	124	19	" "	2.25	3.48			
3	125	19	" "	2.25	3.48	3.48	2.27	100.9
4	126	15	Na_2CO_3		1.39			
4	127	15	"		1.39	1.39		
4	128	15	"	2.25	3.59			
4	129	15	"	2.25	3.65	3.62	2.23	99.1
4	130	19	"		1.29			
4	131	19	"		1.34	1.32		
4	132	19	"	2.25	3.59			
4	133	19	"	2.25	3.59	3.59	2.27	100.9
4	134	15	$K_2C_2O_4$, Na_2CO_3		1.34			
4	135	15	" "		1.34	1.34		
4	136	15	" "	2.25	3.59			
4	137	15	" "	2.25	3.59	3.59	2.25	100
4	138	19	" "	2.25	3.54			
4	139	19	" "	2.25	3.59	3.57	2.23	99.1
5	140	15	Na_2CO_3		2.35			
5	141	15	"		2.19	2.27		
5	142	15	"	2.25	4.44			
5	143	15	"	2.25	4.49	4.47	2.20	97.8
5	144	19	"		2.25			
5	145	19	"		2.25	2.25		

5	146	19	"		2.25	4.44			
5	147	19	"		2.25	4.44	4.44	2.19	97.3
5	148	15	$K_2C_2O_4$,	Na_2CO_3		2.19			
5	149	15	"	"		2.25	2.23		
5	150	15	"	"	2.25	4.44			
5	151	15	"	"	2.25	4.49	4.47	2.24	99.5
5	152	19	"	"	2.25	4.49			
5	153	19	"	"	2.25	4.49	4.49	2.26	100.5
5	154	15	Na_2CO_3			0.79			
6	155	15	"			0.79	0.79		
6	156	15	"		2.25	3.03			
6	157	15	"		2.25	3.03	3.03	2.24	99.5
6	158	19	"			0.79			
6	159	19	"			0.84	0.82		
6	160	19	"		2.25	3.03			
6	161	19	"		2.25	3.14	3.09	2.25	100
6	162	15	$K_2C_2O_4$,	Na_2CO_3		0.90			
6	163	15	"	"		0.79	0.85		
6	164	15	"	"	2.25	3.03			
6	165	15	"	"	2.25	3.09	3.06	2.21	98.2
6	166	19	"	"	2.25	3.09			
6	167	19	"	"	2.25	3.09	3.09	2.24	99.5
7	168	15	Na_2CO_3			1.12			
7	169	15	"			1.12	1.12		
7	170	15	"		2.25	3.37			
7	171	15	"		2.25	3.26	3.32	2.20	97.8
7	172	19	"			1.23			
7	173	19	"			1.18	1.21		
7	174	19	"		2.25	3.48			
7	175	19	"		2.25	3.37	3.43	2.22	98.7
7	176	15	$K_2C_2O_4$,	Na_2CO_3		1.12			
7	177	15	"	"		1.23	1.18		
7	178	15	"	"	2.25	3.37			
7	179	15	"	"	2.25	3.37	3.37	2.19	97.3
7	180	19	"	"	2.25	3.37			
7	181	19	"	"	2.25	3.48	3.43	2.25	100.0

therefore, apparent why there is no interference of the triple phosphate.

From the data presented in table VIII, it is safe to recommend the aeration method with sodium carbonate as the alkali for ammonia determination in normal and Iowa alkali soils. The method complies with the three requirements laid down as fundamental. Closely concordant duplicates are obtained, no appreciable amount of ammonia is given off after fifteen hours aeration, and with the experimental error 100 per cent of any added ammonia was always recovered. The method is eminently practical.

For purpose of comparison, the results obtained by the three methods are assembled in table IX.

TABLE IX.

Soil No.	Ammonia HCl Extraction in Mgs. N	Ammonia MgO distillation in Mgs. N	Ammonia Na_2CO_3 aeration in Mgs. N
1	1.25	3.33	2.03
2	1.24	3.84	2.56
3	0.80	2.67	1.23
4	0.53	2.50	1.39
5	1.23	3.21	2.27

It is seen that there is very little correlation in the amounts of ammonia found by the three methods, except that extraction with the hydrochloric acid gives lower re-

sults, while distillation with magnesia gives higher results than the aeration method. This is to be expected, since we have shown that hydrochloric acid holds back ammonia, while distillation with magnesia effects some decomposition. Contrary to our findings, Kelley and McGeorge[1] report concerning the ammonia determinations in the hydrochloric acid extract, that the "results were very similar to those obtained by direct [magnesia] distillation." Table IX emphasizes the fact that in reporting ammonia determinations in soil, the method used should in all cases be stated.

For the convenience of those who wish to use the aeration method for ammonia in soils, the technique which has been used will be given here in detail. After all the apparatus is at hand, the procedure is as follows: Prepare the absorption bottles as was described above for the Steel emthod. Weigh 25 grams of soil into the 500 c.c. Kjeldahl flasks; add 50 c.c. of ammonia free water and a few drops of a heavy oil to prevent foaming, and then insert the rubber stopper bearing the tube for the entrance and exit of the air. Have the end of the long glass tube come within not more than 3 millimeters of the bottom of the flask. See that no water is between the rubber stopper and the mouth of the flask to collect ammonia. The best way to eliminate this danger is to have the rubber stopper fit very tightly. Now, connect up the whole series, but do not start the pump. As many as fourteen determinations can be run in series. The air, before entering the system, must, of course, be passed through a wash bottle containing dilute sulphuric acid. Now loosen all the rubber stoppers in the Kjeldahl flasks, start the pump, add about 2 grams of sodium carbonate to the flask nearest the pump, shake the flask and then insert the rubber stopper and then take the flasks in succession in just the same way. After a series is once started, the pump should not be stopped. If anything happens to a determination, do not try to remove it. After the aeration has run as long as desired, with the pump still going remove the flasks one by one, starting with the one farthest removed from the pump.

This same apparatus has been used by us for the determination of total nitrogen in soil. The digestion is carried out in the usual way, and the aeration conducted in much the same way as Kober[2] recommends. A water bath, as he advises, was not used, but instead, add about one-third of the required amount of alkali; shake and allow the mixture to cool and then add the remainder of the alkali by the same

1. Bul. Hawaiian Sta. 30: 31. 1913.
2. Loc. Cit.

procedure he uses, except the absence of the water bath. To prevent spattering, the Kjeldahl flask is tilted in a direction perpendicular to a line connecting the two adjacent absorption bottles. As to accuracy and ease of manipulation, we can confirm Kober's statements.

As has been stated, all aeration work reported in this paper is for a current of air of about 250 liters per hour. If a pump is available which moves more air than this, no doubt the time of aeration could be correspondingly lessened.

CONCLUSION.

1. The amount of ammonia extracted by hydrochloric acid is within the limits of our experiments, independent of the strength of the acid and the period of extraction.

2. In the five soils tested, hydrochloric acid removes approximately from 60 to 70 per cent of the ammonia added.

3. The Folin aeration method can advantageously be applied directly to the hydrochloric acid extract.

4. The amounts of ammonia obtained by distillation of the soil directly with magnesia is dependent upon the duration of the distillation.

5. The Steel method of aeration is not suitable for the determination of amomnia in soils.

6. The Steel reagents slowly decompose acetamide.

7. The Folin method of aeration is suitable for the determination of ammonia in soils, for the same result is obtained, whether the reagent acts for a shorter or longer period, and all added ammonia is recovered.

8. In the soils tested there is no interference through formation of triple phosphate.

9. For the soils tested, there was no advantage found in using sodium chloride with the sodium carbonate.

10. Acetamide is not decomposed by 4 per cent sodium carbonate.

11. The results for ammonia obtained by examination of the hydrochloric acid extract are lower, while the results obtained by direct distillation of the soil with magnesia are higher than those obtained by the aeration method. The high results obtained by the former method are due to occlusion of the ammonia y the soil, the nature of which is not clear, and the lower results by the latter method are due to a partial decomposition of the organic material by the magnesia to give ammonia.

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Determination of Amino Acids and Nitrates in Soils

Amino Acids, Ammonia and Nitrates in Manured and Limed Soil

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DETERMINATION OF AMINO ACIDS AND NITRATES IN SOILS.

Amino Acids, Ammonia and Nitrates in Manured and Limed Soil.

By R. S. Potter and R. S. Snyder.**

In view of the fact that no data had ever been obtained on the quantitative relationships of the organic material in the soil by such methods as would preclude a chemical transformation, two things seemed desirable, in continuing the humus investigations[1] at the Iowa Agricultural Experiment Station. First, to attempt the analysis of the organic material of the soil without subjecting it to hydrolysis or other chemical change, and second, to correlate the proportionate quantities of the compounds or classes of compounds found with the kind of soil, its history, treatment, fertility, etc.

As the first step in this investigation, it was decided to determine the amount of amino acid nitrogen for the following reasons: It is through protein material, carried by higher plant, bacterial, mold and animal residues and animal excrements, that much of the nitrogen finds its way to the soil, and any information upon the amount of amino acid nitrogen contained in the soil would be of scientific and practical interest, because the amino acids are the chief products of the chemical hydrolysis and, under certain conditions, of the biologic degradation of this material. It would also be of interest to compare the amounts of amino acid present as such in soil with those found by the long continued boiling of soil with strong acids. There is an abundance of data on this latter point. Lastly, the methods for amino acid nitrogen have been developed to a high degree of accuracy and sensitiveness, and hence definite results may be hoped for.

HISTORICAL.

Following is a brief but complete review of the investigations bearing upon this question.

That part of the nitrogen of the soil is in some kind of amino combination has been recognized for a long time. Baumann found[2] that when soils are boiled with dilute acids 10

*After the senior author took up his present position in the Fall of 1913, many conferences were held with Prof. P. E. Brown, of this Station, and it was mutually agreed that this was a phase of the so-called humus problem which particularly needed study. The authors of this paper wish to express their thanks to Professor Brown for his helpful suggestions and his interest in this work.

1. Jodidi and Collaborators, Iowa Rsch. Bull. 1, 3,, and 9 (1911).
2. Landw. Vers. 32 (1886) 247.

to 20 times as much ammonia is produced as was originally present in the soil. He suggests that the nitrogen of this ammonia might have been present as amino compounds. Berthelot and Andre[3] found that the amount of ammonia and other soluble nitrogenous products produced from soil by the action of acids was directly proportional to the strength of the acid, the duration of its action and the temperature at which it acted. From their results, they concluded that the greater part of the soil nitrogen existed in insoluble amides, and that acids split these into ammonia and soluble amides.

Sestini[4] upon treating "humus acid" with strong nitrous acid obtained a "significant" evolution of nitrogen, and from this fact states that his humus contains amino acid. Since it has been shown that unhydrolysed protein, when treated with nitrous acid gives nitrogen[5], he was not justified in the conclusion he drew. Using the same method, Dojarenko[6] reports from 1.01 to 1.34 per cent of amino acid nitrogen in humus preparations from various soils, but since he boiled his humus with dilue hydrochloric acid, his results cannot be considered as representing the amounts actually present in the soil.

In the last decade Shorey[7], Suzuki[8], Jodidi[9], Lathrop and Brown[10], Robinson[11] and Kelley[12] have subjected the solution obtained by boiling the soil, or its alkali extracted materials, with more or less strong acids, to the scheme of separation of the nitrogenous constituents into amids, basic and non-basic compounds as outlined by Hausmann[13] and Osborne and Harris.[14] In round numbers 25% amide, 10% basic, 65% of non basic nitrogen was found and Jodidi showed that of the basic nitrogen a "large part" consisted of diamino acids and approximately 75% of the non basic nitrogen was made up of monamino acids. Suzuki also subjected a sample of Kahlbaum's "humic acid" to the Fisher[15] ester method of amino acid separation, and obtained several of the amino acids which had previously been separated from pure hydrolysed proteins.

In recent years, Schreiner and Shorey have isolated, among a host of other organic compounds, the amino acids histidine[16],

3. Compt. rend., 103 (1886) 1101.
4. Landw. Vers., 51 (1899) 153.
5. Schiff, Ber. 29 (1896) 1354.
6. Landw. Vers. 56 (1902) 311.
7. Agr. Invest. Hawaii in 1905, Rept. Chem., p. 34.
8. Bull. Col. Tokyo, 7 (1907) 513.
9. Mich. Tech. Bull., 4 (1909); Iowa Rsch. Bull., 1, 3 and 9 (1911).
10. J. Ind. Eng. Chem., 3 (1911) 357); Am. Ch. Soc., 27 (1910) 396.
11. J. Am. Chem. Soc., 33 (1911) 564.
12. Ibid., 36 (1914) 429.
13. Z. Physiol. Ch., 27 (1899) 5.
14. J. Am. Chem. Soc., 25 (1903) 223.
15. Z. Physiol. Chem., 33 (1901) pp. 151, 412.
16. J. Biol. Chem., 8 (1910) 382.

arginine[17] and lysine[18] from a weak alkali extract of soil, and there is every reason to believe that these compounds were present in the soil as such. The investigators made no attempt to use quantitative methods. Quite recently Kelley and Thompson[19] subjected the so-called humus solutions of several soils obtained by treating 40 g. of soil for 48 hours with 2 liters of 3% of sodium hydroxide solution, to the Osborne and Harris scheme of analysis and obtained, as an average of the soils tested in per cent of total soil nitrogen, 17% amide, 6% basic and 34% of non basic nitrogen. It has been shown by the writers[20] that soil treated with 1% sodium hydroxide gives a continuous evolution of ammonia for at least the first 19 hours, which was as long as the test was run, and that the ammonia given off in this time was in considerable excess of that present in the soil as determined by aeration with sodium carbonate, or even by distillation with magnesia. Therefore, it would seem that the values they obtained for the amide nitrogen were too high.

Since the relative completeness of the precipitation of the basic nitrogenous compounds with phosphotungstic acid depends largely upon their concentration in the solution, reserve must be exercised in drawing conclusions from the figures reported by Kelley and Thompson for the basic nitrogen, as no data are given showing to what volume of solution the phosphotungstic acid was added.

The highest practical concentration they could have used was the concentration of about 1500 c. c. of the extract to 200 c. c. They could not have gone much farther than this, due to interference of sodium chloride crystallizing out. If it is assumed that this was what was done, in the 200 c. c. from the extract of the eight soils with the highest and lowest amounts of nitrogen, there would have been 0.202 g. nitrogen and 0.0201 g. nitrogen respectively, and an average of 0.084 g. nitrogen for the eight soils. When 200 c. c. solutions containing arginine, histidine, lysine and cystine are precipitated by phosphotungstic acid, it has been shown[21] that 0.0101 g. nitrogen from these compounds is not carried down, and hence this correction would be added to the amounts of nitrogen found in such a precipitate. While it is probable that only a small proportion of the precipitate obtained with phosphotungstic acid by Kelley and Thompson was due to the above mentioned amino acids, yet it is clear that a large

17. Ibid.
18. U. S. Dept. Agr. Bur. of Soils, Bull., 88 (1913).
19. J. Am. Chem. Soc., 36 (1914) 438.
20. J. Ind. Eng. Chem., 7 (1915).
21. J. Biol. Chem., 10 (1911) 32.

part of any which might have been present would be in the filtrate.

It is entirely possible that there were many other basic compounds only partially precipitated by the reagent, and of course, this would seriously modify the results, especially for the more dilute solutions. Then too, the hydrolysis involved in concentrating the 1500 c. c. to 200 c. c. would be serious, and if such a concentration were not made, the solutions were of a still greater dilution, and hence the effect of the solubility of the phosphotungstates all the greater. Therefore, it is probably true that the figures obtained by Kelley and Thompson are too low for the so-called basic and too high for the non-basic nitrogen.

After most of the experiments carried out in connection with this investigation were completed, there appeared a paper by Chardet[22] in which he reports the analysis of four soils for total, amino acid, and ammonia nitrogen. The results he obtained are given in the following table:

SOIL	Total N. in % of Soil	Ammonia N. in % of Total N.	Amino Acid N. in % of Total N.
1	0.22	6.5	66
2	0.28	8.0	52
3	1.65	6.0	68
4	2.10	12.0	49

The details by which he obtained the above figures he gives as follows: The soil was treated with 2 per cent sodium hydroxide in the cold, the liquid was decanted and acidified with hydrochloric acid and filtered. To the filtrate a few c. c. of silver nitrate solution was added and upon filtration, a clear, colorless solution was given. A 10 per cent barium chloride solution was added to this filtrate which was then made neutral to phenolphthalein with sodium hydroxide and the resultant precipitate filtered off. The solution obtained was aliquoted, part being taken for the determination of ammonia by distillation with magnesium oxide and the other used for the estimation of amino acid nitrogen by the method of Sorenson[23]. This method, following the details of Chardet, was applied to various soils by the writers, but in no case was an appreciable amount of amino acid nitrogen found. His results in other ways do not agree with our experience with soil organic matter. In the work done in this laboratory, not more than 35 per cent of the soil nitrogen was ever found in the filtrate obtained by acidification of the alkali

22. Rev. gen. Chim., 17 (1914) 137.
23. Z. Physiol. Chem., 60 (1909) 1; 64 (1910) 121.

extract. Kelley[24] in his work found as an average of eight soils only 26 per cent of the total nitrogen in such a filtrate. The only explanation of Chardet's results which can be offered is that the formol he used contained acid, as it usually does, and since he made no note of it, it is probable that no correction was made for this.

It is, therefore, seen that except for the work of Chardet, which has not been confirmed, the only proof there is of the presence of amino acids in the soil is the work of Schreiner and Shorey. Until quite recently, there was no method sufficiently sensitive to permit of the accurate determination of the amino acids in the soil. The copper method of Kober[25], however, the details of which are given below, being capable of detecting with considerable accuracy one part of amino acid nitrogen in 500,000 parts of solution, has been found very suitable for soil work. By this method 5 to 30 parts of amino acid nitrogen per million parts of soil have been found, so it can easily be seen how the earlier methods would be entirely unsuited. The formol titration method of Sorenson[26] has been shown by Loeb[27] to be accurate to only one part in 1600, while the Van Slyke[28] method has been shown by its originator to be accurate to one part in 10,000, and besides is specific for the amino group, not for amino acids.

THE KOBER METHOD FOR AMINO ACID NITROGEN

It is not the place here to discuss at length the theoretical basis of the Kober method for amino acid nitrogen, as it is fully set forth in the papers by Kober[29] and collaborators. Suffice it to say that by it one may determine the A and B amino nitrogen in A and B amino acids and such derivatives of these compounds as have one hydrogen atom of the amino group replaced by "any positive radical or group as CH_3-, an amino acid, or a combination of amino acids. This includes all the amino acids thus far isolated from proteins, all the polypeptides and the peptones." In solutions having a hydrogen ion concentration of from $10^{-7.07}$ to $10^{-8.8}$, the compounds cited above dissolve quantitatively cupric hydroxide in the proportions indicated by the following relations: (Amino acid)$_2$Cu; (Peptide)$_1$Cu.

It should be particularly pointed out that in both the amino acids and their derivatives it is only the nitrogen which is in the amino group in the A and B position with reference to

24. J. Am. Chem. Soc.,36 (1914) 438.
25. J. Am. Chem. Soc., 35 (1913) 1546.
26. Loc. Cit.
27. Ber., 46 (1913) 696.
28. J. Biol. Chem., 7 (1910).
29. Loc. cit. also J. Biol. Chem., 13 (1912) 1; Am. Chem. J., 48 (1912) 383.

the unsubstituted carboxyl group, which is found by this method. Hence in a solution containing a peptide made up of four monamino acids on analysis the nitrogen obtained would represent one-fourth of the total nitrogen.

If to a solution of the above hydrogen ion concentration containing a mixture of the copper complexes of the amino acid and the peptides sufficient barium hydroxide be added to give a 0.06 N. solution of the alkali, a definite fraction (86.5%) of the copper from the free amino acid complexes is precipitated. Upon this is based the procedure of the method for the separate determination of the free amino acid nitrogen and polypeptide amino nitrogen. There are many other substances that dissolve cupric hydroxide, but it has been shown that these can be removed quantitatively with ammoniacal lead acetate.

EXPERIMENTAL.

The technique of the Kober method as recommended by its originator was followed, with some modifications, in all its essentials in this work. The cupric hydroxide was prepared in an ice mixture and centrifuged instead of filtered as Kober recommends. The buffer solution was titrated from time to time, and it was found to hold its original strength. The thiosulphate solutions were standardized from time to time against standard iodine solutions. S. and S. no. 589, Blue Ribbon filter paper was used throughout, instead of S. and S. no. 590, as recommended by Kober. The no. 590 paper, although more rapid, often caused trouble. Any other deviations from the original method are cited in the proper place.

It is a well known fact that dilute acids in the cold dissolve only a small part of the soil nitrogen, yet it is known that 1% HCl dissolves some organic nitrogen[30], hence it was thought possible that any amino acids present would be dissolved. Of a soil which contained 0.181 per cent nitrogen, 150 g. was shaken with 300 c. c. of 0.2 N. hydrochloric acid for one hour. The mixture was poured upon a paper filter, and 80 c. c. of the clear filtrate was transferred to a 100 c. c. flask; 7 c. c. of a saturated solution of lead acetate and 7 c. c. of ammonia (sp. gr. 0.90) was added, and water to complete the volume to 100 c. c. The mixture was thoroughly shaken and then filtered. To 75 c. c. of the filtrate, 25 c. c. of a saturated solution of barium hydroxide was added, and the ammonia removed by boiling at a pressure of about 25 mm.*

30. Kelley and Thompson, Loc. Cit.

*After this procedure Kober found it necessary to remove lead either with potassium sulphide or sulphate. In our experience, the lead is entirely precipitated by the ammonia. This is in harmony with the statement found in text books that lead hydroxide is insoluble in excess of ammonia.

The liquid was then made up to 75 c. c., the barium carbonate filtered off and 50 c. c. of the filtrate transferred to a 100 c. c. flask. The solution was then made barely alkaline to phenolphthalein, 40 c. c. of "buffer" solution added, and after cooling for about two hours or more in the ice box, one c. c. of a cold suspension of cupric hydroxide in water was pipetted in, the whole vigorously shaken, water added to the mark and the mixture filtered through S. and S. no. 589 Blue Ribbon filter paper.

Fifty c. c. of the filtrate showed no trace of copper. Other soils treated in the same way gave the same result. Upon adding a small amount of alanine to some soils and then extracting with 0.2 N. hydrochloric acid in the same way, no amino acid nitrogen was found. This result is somewhat in harmony with the fact that only from 60 to 70% of small amounts of ammonia added to the soil are extracted with 0.2 N. acid.[31]

Dilute alkali is known to dissolve more organic matter from the soil than any other of the milder reagents, therefore 2 per cent sodium hydroxide was next tried as the extraction agent and was found to be quite satisfactory. Much the same procedure was used here as for the acid extraction, except that 600 c. c. of the alkali was used with 150 g. soil. It is almost impossible to filter this extract by any of the ordinary methods, so it was cleared by centrifuging in the machine described in a bulletin from this station*. After whirling in this machine for a few minutes, the clear but dark colored solution was drawn off through the silver tube. Eighty c. c. of this solution was approximately neutralized with 1-1 HCl and then the same procedure carried out that was used in the acid extract up through the filtering off of the excess copper hydroxide.

Although with all the soils tested copper was always found in this filtrate, yet there was present some reducing substance which took up the iodine liberated by adding potassium iodide. Hence, instead of titrating directly 50 c. c. was placed in a beaker, acidified slightly with nitric acid, heated to boiling and bromine water added until a permanent color was given. The solution was then boiled down to about 10 c. c. and to make sure the last traces of free bromine were given, 30-40 c. c. water added and again boiled down to 10 c. c. The solution was cooled, neutralized with sodium carbonate, a very slight excess of 1% hydrocholoric acid added and after the addition

31. Potter and Snyder, Loc. Cit.

*Stevenson, Wells and Coover. Iowa Bull., 124 (1911). A glass tube was originally recommended for drawing off the solution. We are indebted to Prof. R. E. Smith for the suggestion of the use of a siver tube which has been found much more satisfactory.

of the potassium iodide and starch solution, the free iodine was titrated with 0.001 N. sodium thiosulphate solution. Each c. c. of this solution equals 0.0280 mg. of amino acid nitrogen, or 0.0140 mg. peptide amino nitrogen. For the determination of the free amino acid nitrogen, 40 c. c. of the solution was placed in a Erlenmeyer flask; following the adding of 8 c. c. of 0.360 N. barium hydroxide, which precipitates 86.5 per cent of the free amino acid copper, the mixture was allowed to stand for 15 minutes, filtered and, after thoroughly washing the precipitate, washed through the filter with warm 1 per cent hydrochloric acid and then with water. The solution obtained was evaporated to a small volume, neutralized with sodium carbonate, slightly acidified with acetic acid and then, after the addition of potassium iodide and starch, titrated with 0.001 N. sodium thiosulphate. Here as above, 1 c. c. of the thiosulphate equals 0.0280 mg. amino acid nitrogen. It was pointed out by Kober that if polypeptides of six or more conjugated amino acids are present, they will prevent the precipitation of the free amino acid copper. In all the soils so far analyzed, it has been found that from 60 to 85 per cent of the copper was precipitated by the barium hydroxide, so the interference from that standpoint is probably negligible.

As a check upon this work, analyses were run on some pure glycerine and alanine solutions, and also known amounts of these substances were added to soil and the increase in amino acid nitrogen found. For the analysis of the pure amino acid solutions, the indicated amounts obtained by aliquoting strong solutions were placed in 100 c. c. flasks, made up to about 80 c. c. with sufficient sodium hydroxide to give a 2 per cent solution of the alkali and from then on run exactly as was the soil. The amino acids were added to the soil by aliquoting the same solutions, and then sufficient sodium hydroxide solution to give 600 c. c. of 2 per cent alkali, and after shaking two hours and centrifuging, 80 c. c. of the extract were taken and the regular procedure carried out. The results are given in the following table and are calculated in every case to the amount of amino acid nitrogen in the 80 c. c. of solution or extract.

From results set forth in table I, it is seen that by the analysis of the pure glycine 92.4 per cent of the theoretical quantity was found, while in the case of the alanine 94.6 per cent was found. Since the samples used were Kahlbaum's C. P. material, and since practically the theoretical amount of nitrogen was found in each case by the Kjeldahl analysis, the material is, no doubt, very close to 100 per cent pure. When it is considered how small amounts were dealt with in each case, the low percentages obtained are not surprising. In solutions of glycine containing approximately the same amounts,

Kober found in one case 94.8 per cent and in another 95.7 per cent of the theoretical quantity. The recovery of the 87.5 per

TABLE I

SUBSTANCE	AMINO NITROGEN IN MG.						Per Cent Amino N. Recovered
	Added	Found	Average	Per Cent Found	Recovered	Average	
Glycine	0.224	0.203					
Glycine	0.224	0.210	0.207	92.4			
Soil A		0.168					
Soil A		0.174	0.171				
Soil A and Glycine	0.224	0.370			0.199		
Soil A and Glycine	0.224	0.364	0.367		0.193	0.196	87.5
Alanine	0.224	0.208					
Soil A and Alanine	0.224	0.216	0.212	94.6			
Alanine	0.224	0.375			0.204		
Soil A and Alanine	0.224	0.380	0.378		0.209	0.207	92.4

cent of the added glycine and 92.4 per cent of the added alanine, when the many operations through which the soil solutions must pass are considered, seems, on the whole, not unsatisfactory.

In order to determine if the amount of amino acid nitrogen was increased by continued treatment with the alkali, samples of two soils were shaken with 2 per cent sodium hydroxide for varying lengths of time. The results are given in table II.

TABLE II

SOIL	Time Shaken in Hours	Amino Acid N. in p. p. m. (a) of Air Dry Soil	SOIL	Time Shaken in Hours	Amino Acid N. in p. p. m. of Aiur Dry Soil
A	1	8.4	C	1	22.4
	2	8.1		2	23.0
	1	7.8		1	23.0
	2	7.8		2	23.2
	4	8.4		4	22.4
	4	8.1		4	23.2
	6	8.4		6	23.2
	6	7.8		6	23.2

(a) P. p. m.: Parts per million.

The results show that within the six hours for the soils tested, there is neither any decomposition of amino acids or degradation of more complex material to give amino acids. Of course, another possibility is that the rate of decomposition of the amino acids is just equal to their rate of formation, but that is hardly possible. These results are in harmony with the known stability of amino acids and of protein material as far

as amino acid production is concerned, in the presence of weak alkali.

If a solution is found to contain a certain amount of amino nitrogen of free amino acids, then the increase in amino acid nitrogen after complete hydrolysis of the solution will give the total amount of peptide nitrogen originally present. This last procedure has been carried out in the case of two soils. The hydrolysis was carried out by boiling the solutions for 15 hours with 30 per cent sulphuric acid in flasks provided with reflux condensers. The results are given in table III.

TABLE III

Soil	Nitrogen in p. p. m. Air Dry Soil		
	Total Amino Acid	Free Amino Acid	Total Peptide N.
A	8.1	5.6	32.6
	7.8	5.3	30.4
B	23.0	15.0	30.0
	23.2	14.4	31.8

It is seen that while there is quite a difference in the amount of total amino acid nitrogen in the two soils, yet there is practically no difference in the amount of total peptide nitrogen. The relationship of these quantities will be investigated more fully in the future in this laboratory.

The consistency with which the amount of amino acid nitrogen is so small in the soils reported above points to the fact that, as with ammonia, there is not much tendency for it to accumulate in soils. This is, perhaps, to be expected from the fact that amino acids are known to be very good nutritive media for bacteria.[32] It has also been shown by Jodidi[33] that those amino acids made up of aliphatic nuclei, when incubated at room temperature for from six to ten days with soils, containing 18 per cent moisture in amounts varying from 0.1 gr. to 0.5 gr. amino acid per 50 g. soil increased the amount of ammonia nitrogen in the soil by from 44 per cent to 74 per cent of the amount of amino acid nitrogen added. The amino acids tyrosine and phenylalanine, containing carbocyclic groups, under similar conditions give rise to approximately 20 per cent less ammonia. As pointed out by Jodidi, the actual production of ammonia might have been really greater, because part of the ammonia produced might have been nitrified. No doubt, also part of the nitrogen was assimilated by bacteria. The amount of amino acid added to

32. Czpek, Hofmeister's Beit., 1 (1902) 538; 2 (1902) 557; 3 (1902) 47; Emmerling, Ber., 35 (1902) 2289.
33. Iowa Research Bull., 9 (1912).

the soil by Jodidi was far in excess of that actually present in any soil which we have examined, yet since he found the percentage transformations was about the same whether he added 0.1 g. or 0.5 g. of amino acid, no doubt the rate of transformation of still smaller amounts would be about the same.

It is well known that the action of bacteria and boiling acids upon proteins gives rise to about the same products, namely, proteoses, peptones, peptides and amino acids, but as pointed out above, the action of bacteria does not stop with amino acids. Another important factor to be taken into account when considering the transformation of soil nitrogen is the assimulation of the nitrogen by bacteria. While data are lacking on the chemical constitution of bacteria usually present in soil, yet their composition is probably not far different from that of some of the pathogenic organisms. It has been shown by Cramer,[34] for instance, that *Spirillum cholerae* has a protein content varying from 45 per cent to 65 per cent, according to whether it was grown on a medium poor or rich in protein material. Therefore, in soil there are two opposing tendencies, namely, the degradation of the complex nitrogenous material into its constituent parts and subsequent ammonification and nitrification of these parts, and then the assimilation of the degradation products by the microflora of the soil to give again material less complex than for originally.* Of course, there are other changes, such as assimilation of the nitrogen from the atmosphere and denitrification, but these and other changes need not be entered into here.

It is a well known fact that the introduction of large amounts of fresh organic matter in soils causes a great depression or the complete disappearance of nitrates therefrom, and also prevents any appreciable formation and accumulation of nitrate nitrogen. Various explanations have been offered to account for this, such as denitrification, assimilation, or the depression of nitrification because of accumulated intermediate products of degradation. Whether there is a continuous production of nitrates which are subsequently assimilated, or whether the organisms get their nitrogen from the ammonia or amino acids or other compounds cannot be definitely stated, but in well aerated soils not too wet, one would certainly expect nitrification to be taking place, and for the same reasons none or only a negligible amount of denitrification. The use of the term "acid soluble" by Jodidi[35] in the following statement has lead to some confusion on this question: "The

34. Archiv. f. Hygiene, 12, 157; 13, 76; 16, 171.

*It has been shown by Bierma (Centra. Bakt. Parasitenk II Abt. 23 (1909) 672) that amino acids, as well as ammonia are assimilated by soil bacteria.

35. Iowa Research Bull., 8, 121 (1911).

principal portion of the acid soluble organic nitrogen contained in the soil herein investigated is made up of acid amides, monamino acids and diamino acids."

The Kober method for amino acids, as we have applied it to soils will give definite information as to the amounts of amino acids actually present in soil, and will, therefore, show whether under various conditions there is any tendency for these products to accumulate in soil. Interest in the question is also raised by the accumulating evidence that green plants may utilize amino acids[36] for their nitrogen nutrition. While it is not thought that green plants would utilize an appreciable amount of amino acid in the presence of nitrates and ammonia, yet it is possible that in soils temporarily deprived of nitrates, the amino acids, as well as the ammonia, might be used by the plant.

It was to study the problems suggested by the above discussion that the following experiment was performed:

Several hundred pounds of soil A, which was acid to litmus and showed a lime requirement of 3800 pounds of calcium carbonate per 2,000,000 pounds soil by the Veitch[37] method and which contained 0.151 per cent of nitrogen, were procured. The sample was air dried, shaken through a four mm. sieve and very thoroughly mixed. Twenty pot experiments, each pot containing 30 pounds of air dry soil, were run with this soil with treatments indicated in table IV.

TABLE IV

Pot No.	Treatment, in Tons per 2,000,000 lbs. of Soil
1 and 2	Nothing
3 and 4	4 of $CaCO_3$
5 and 6	10 of Stable Manure
7 and 8	20 of Stable Manure
9 and 10	30 of Stable Manure
11 and 12	50 of Stable Manure
13 and 14	4 of $CaCO_3$—10 of Stable Manure
15 and 16	4 of $CaCO_3$—20 of Stable Manure
17 and 18	4 of $CaCO_3$—30 of Stable Manure
19 and 20	4 of $CaCO_3$—50 of Stable Manure

The manure used was collected from the stalls of horses and was made up of about equal parts of the droppings and of the wet straw. It was cut up and mixed while still moist and added to the soil in that condition. The object of adding the moist manure was to avoid too great a modification of the bacterial flora. Because of the condition of the manure, it was impossible to obtain a perfectly uniform mixture, or to mix it with the soil as well as was desirable.

36. Molliard, Bull. Sec. botan. France, 57, 541-547 (1909), Hutchinson and Miller, J. Agr. Sci., 4, 282.
37. J. Am. Chem. Soc., 24 (1902) 1120.

Manure gathered as above was analyzed for total amino acid nitrogen, ammonia nitrogen and total nitrogen. For the amino acid nitrogen 100 grams was stirred with 400 c. c. of 1 per cent sodium hydroxide for a few minutes, the coarser particles were strained out and the liquid was then filtered through a folded, dry filterer. Aliquots were taken for duplicate determinations and the solution was then carried through precisely the same procedure as the soil extract.

For the ammonia determination, 50 g. of the manure was placed in 500 c. c. Kjeldahl flasks, 200 c. c. of water and about 5 grams of sodium carbonate added and the ammonia determined by aeration. Aeration was continued until practically no more ammonia was being given off.

The total nitrogen was determined in 2 g. samples obtained by drying and grinding a larger sample. The results are given in table V.

It should be pointed out that the determinations were made on separate samples. That is, ammonia and amino acids were not determined on the same sample. The discrepancies of the resuts illustrated the non-uniformity of mixing. The results are calculated to the wet basis, that is, just as it was added to the soil.

TABLE V

NITROGEN IN P. P. M. WET MANURE

Amino Acid	Average	Total Ave.	Ammonia	Total Ave.	Total	Average	Total
30.0			140		5000		
32.0	31.0		161		5200	5100	
25.1			155		4670		
24.3	24.7				4920	4795	
32.1					5250		
33.1	32.6	29.4		152	5670	5460	5118

All of the pots were maintained as closely as possible at 20 per cent moisture by replenishment of the evaporated water every two or three days with distilled water. The experiment was started on May 15, 1914, the first sampling was made on May 18, and then every two weeks until in all five samplings had been made. On each of the samples taken, amino acid, ammonia and nitrate nitrogen were determined by the following methods:

Methods

Amino Acid Nitrogen—At the first sampling, the moisture was immediately accurately determined. Enough of the fresh soil to give 150 grams of dry soil was put in a bottle and suffi-

cient water and 4 per cent sodium hydroxide added to give 600 c. c. of 2 per cent alkali; and after shaking two hours, the total amino acid nitrogen was determined. Then the fresh soil, as soon as the soil had been withdrawn for the determinations of the amino acid nitrogen in the wet soil, was quickly dried with the aid of an electric fan. The total amino acid nitrogen was then determined in the air dry soil in the usual way. The results using the fresh soil and the air dried soil were practically the same, so for the remaining samplings, air dried soil was used. The dissolved copper was titrated with 0.001 N. sodium thiosulphate. Duplicate determinations were always run.

Ammonia Nitrogen.—Seventy-five g. air dried soil, 200 c. c. distilled water, 10 g. magnesia and a small piece of paraffin were placed in a copper flask and distilled, 150 c. c. of distillate being collected in 0.1 N. acid.* The results by this method are, no doubt, high, but conditions were kept as nearly constant as possible, so the results are probably at least comparable.

Nitrates.—The phenoldisulphonic acid method as modified by Chamot[38] and collaborators was used throughout. As is well known, this method gives somewhat low results with higher amounts of nitrates. One hundred g. of soil and 2 g. of calcium carbonate were shaken for about a half hour with 200 c. c. of water and immediately filtered and an aliquot of the solution so obtained immediately evaporated to dryness; treated wth the phenoldsulphonic acid reagent and after dilution potassium or sodium hydroxide** added until the maximum color was produced.

C. B. Lipman and Sharp[39] recommended the use of quicklime for the flocculating agent in nitrate determination. As they state, J. G. Lipman and P. E. Brown in their laboratory manual, "Soil Bacteriology", give directions for the use of lime for such purposes, but Dr. Brown states that he always has used lime in the form of carbonate. It was thought desirable, therefore, to compare the action of these two flocculating agents. At the same time, parallel tests were made with the aluminum reduction method, using in all essential principles the modification of Burgess.[40] The aeration apparatus described elsewhere[41] was made use of. The solutions were

*Since the experimental part of this paper was completed, the authors have worked out a method for the determination of ammonia in soils (Loc. Cit.) which is much more satisfactory than the magnesia method.

38. J. Am. Chem. Soc. 33 (1911) 381.

**The original directions call for potassium hydroxide, but we have found sodium hydroxide equally satisfactory.

39. Univ. Cal. Pub. Agr. Sci., 1 (1912) 21.

40. Univ. Cal. Pub. Agri. Sci., 1 (1913) 51.

41. Loc. Cit.

transferred from the casseroles to the 500 c. c. Kjeldahl flasks, aluminum strips added and then the flasks were connected to their respective absorption bottles which contained a suitable quantity of 0.02 N. acid in 200 c. c. of water. After standing 11-15 hours, the series was connected up and aerated and the excess acid titrated with 0.02 N. alkali, using alizarin red as the indicator. If the apparatus is at hand, this procedure takes much less time than that used by Burgess.

Solutions of pure potassium nitrate were first experimented with. In each case, the respective flocculating agent was shaken with the solution at the rate of 2 g. per 100 c. c. of solution. Where both methods were used, aliquot parts of the same filtrate were used. The results given are averages of closely agreeing duplicates.

The results show that while the use of calcium oxide gives low results with pure nitrate solutions by the colorimetric method, yet practically the entire amount is found by the reduction method.

Comparative experiments were next carried out on soils with high and low nitrate content, some with known amounts of nitrate added. In all cases, one part of soil was shaken for a half hour or less with two parts of water, and the floc-

TABLE VI

FLOC. AGENT	NITRATE IN P. P. M.		
	Present	Found Color	Found Red
$CaCO_3$	1	0.97	.....
CaO	1	0.40	.....
$CaCO_3$	10	9.00	9.94
CaO	10	6.60	9.66

culating agent was added at the rate of 2 g. per 100 g. soil. The results given in table VI were all found by the colorimetric method.

The important point brought out by the above results is that calcium oxide gives decidedly low results with soils containing a small amount of nitrogen.

The reduction and the colorimetric method were tried with soils which had been incubated 30 days with 0.150 g. ammonium sulphate per 150 g. soil. The extraction of the nitrate was carried out as usual. The results given represent the amounts found in duplicate incubations and not duplicates on the same incubation. For the two methods, aliquot parts of the same filtrate were used.

The above results show, in harmony with the experience of

TABLE VII

Soil No	Lab. No.	NITRATE IN P. P. M.				
		Floc.	Added	Found	Recovered	% Recovered
69	.....	$CaCO_3$	None	6.7	.....	.....
.....	.....	$CaCO_3$	3.0	9.6	2.9	97.0
.....	.....	CaO	None	2.0	.....	.....
.....	.....	CaO	3.0	2.9	0.9	30.0
268	.....	$CaCO_3$	None	14.7	.....	.....
.....	.....	$CaCO_3$	3.0	17.6	2.9	97.0
.....	.....	CaO	Nont	7.6	.....	.....
.....	.....	CaO	3.0	7.6	None	0 0
355	.....	$CaCO_3$	None	2.2	.....	.....
.....	.....	$CaCO_3$	3.0	5.0	2.8	93.0
.....	.....	CaO	None	1.6	.....	.....
.....	.....	CaO	3.0	3.1	1.5	50.0

TABLE VIII

Soil No.	Floc Agent	NITRATE IN P. P. M.	
		Color	Red
C	$CaCO_3$	152	183
	$CaCO_3$	152	176
	CaO	135	Lost
	CaO	139	175
D	$CaCO_3$	145	171
	CaO_3	149	170
	CaO	139	169
	CaO	Lost	169

others, that the colorimentric method gives somewhat lower results than the reduction method. With the former method, calcium oxide produces somewhat lower results than calcium carbonate, while there is no apparent difference by the reduction method.

From the data which are presented, one is forced to the conclusion that with soils low in nitrates the use of calcium carbonate is to be preferred over calcium oxide, and when the colorimetric method is to be used, the carbonate is always better. The carbonate in all cases yields as clear and as nearly colorless a solution as the oxide. As to the reason for the low results by the colorimetric method with the calcium oxide, we are not prepared to answer. While the subject is, no doubt, worth more complete investigation, since the calcium carbonate has proven to be entirely satisfactory, we have not seen fit to carry the subject any further.

The Results of the Pot Experiment

Before entering into a discussion of the results from the first sampling, the amounts of amino acids present immediately

after addition of the manure will be computed. They will be computed on the air-dry basis, thus making them comparable to the results found by analysis of the samplings.

Amino Acid Nitrogen in P. P. M. Air-Dry Soil

Pots Nos.	
1, 2, 3, 4	10.8 (by analysis)
5, 6, 13, 14	11.0 computed
7, 8, 15, 16	11.2 computed
9, 10, 17, 18	11.4 computed
19, 20, 21, 22	11.8 computed

It was not thought best to include these values in the curves which are given later, as the differences between the amino acid results as computed and those found on the third day are so small that any apparent difference might be due to analytical errors rather than actual change. It would be manifestly inadmissable to compute the ammonia values as was done for the amino acid nitrogen, for the ammonia values as found in the soil by the magnesia method are undoubtedly too high; hence to add the value so obtained to that found to be actually present in the manure, would be in no way comparable to amounts found later for the soil and manure by the magnesia method. It would be no more permissible to find the ammonia in the manure by the magnesia method and then compute values for the various pots, for the organic matter in the manure and soil would not decompose to the same extent when together as when separate.

No nitrates were present in the manure, and less than 0.5 p. p. m. were present in the soil at the start.

The results obtained on the first sampling, three days after making up the pots to the proper moisture are given in table IX.

The results are given in table IX with respect to the amino acid show very little difference between the limed and the unlimed soils, while the amount rises with increasing application of manure. There is a slightly lower amount in the pots with no manure and with the smaller applications than the computed original values, while the more heavily manured soils show slightly higher values than the calculated one. The differences are, however, so small, that no conclusions should be drawn.

The ammonia results are quite interesting. All the soils show greater amounts than the check pots, and the unlimed manured pots are higher with greater amounts of manure, while the limed manured pots show, in general, decreasing ammonia content, with increasing applications of manure. These results are somewhat surprising when it is considered that the limed unmanured soils have more ammonia than the

TABLE IX

Pot No.	NITROGEN IN P. P. M. AIR DRY SOIL					
	Amino Acid	Ave.	Ammonia	Ave.	Nitrate	Ave.
1	10.2	.....	50.4	.....	6.4	.....
2	10.0	10.1	46.3	48.3	6.4	6.4
3	10.5	.....	56.0	.....	6.4	.....
4	9.5	10.0	51.8	53.9	6.4	6.4
5	10.6	.....	50.4	.....	4.4	.....
6	10.6	10.6	60.2	55.3	1.8	3.1
7	10.6	.....	65.8	.....	2.2	.....
8	11.0	10.8	56.0	60.9	2.2	2.2
9	12.9	.....	62.3	.....	0.8	.....
10	Lost	19.9	65.5	64.4	0.8	0.8
11	11.8	.....	70.7	.....	0.6	.....
12	14.0	12.9	70.0	70.4	0.6	0.6
13	10.0	.....	67.2	.....	3.2	.....
14	10.6	10.3	53.2	60.2	3.2	3.2
15	10.7	.....	57.4	.....	1.3	.....
16	11.2	11.0	60.2	58.8	2.6	1.95
17	10.9	.....	46.9	.....	0.84	0.82
18	11.5	11.2	48.3	47.6	0.8	.....
19	12.8	.....	49.0	.....	1.2	.....
20	12.2	12.5	57.4	53.2	0.8	1.0

check pots. Any answer must necessarily be purely speculative, yet no doubt, lime enhances the action of the bacteria introduced with the manure, which tends to diminish the ammonia, either by direct assimilation or nitrification and subsequent assimilation. In connection with this, it should be pointed out that by the application of wet manure, a large amount of urea, which is readily ammonified, is introduced.

As to nitrates, there are no differences which could not be attributed to analytical errors between the limed and unlimed soils. There is a striking decrease in the amount of nitrate nitrogen with increasing amounts of manure. Two explanations of this suggest themselves, namely, denitrification and assimilation. Which of these is correct or whether it is a combination of the two, it is not possible to state.

From the results given in table X, which were obtained two weeks after the first sampling, it is seen that there is a decided decrease in the amount of the amino acid nitrogen. The tendency is, except for the unmanured soils, for the unlimed to run higher than the limed, with only slightly more amino acid nitrogen in the heavily manured soils than in those with no manure.

As to the values for the ammonia, there is a tendency for them to be not so widely divergent as on the first sampling. The limed manured soils are slightly above the unlimed manured, while the limed unmanured are below the check.

The nitrates are present in each case in slightly higher amounts than on the first sampling, and the same differences hold.

TABLE X

Pot No.	NITROGEN P. P. M. AIR DRY SOIL					
	Amino Acid	Ave.	Ammonia	Ave.	Nitrate	Ave.
1	5.04	.....	44.4	.....	18.9	.....
2	6.44	5.74	47.6	46.0	27.7	23.3
3	6.72	.....	38.8	.....	18.9	.....
4	5.60	6.16	44.8	41.8	21.6	20.3
5	7.56	.....	37.8	.....	8.0	.....
6	8.96	8.31	42.3	40.0	9.6	8.8
7	7.28	.....	40.6	.....	5.6	.....
8	8.12	7.70	47.6	44.1	4.0	4.8
9	8.68	.....	47.9	.....	2.2	.....
10	8.12	8.40	39.8	43.9	2.2	2.2
11	8.68	.....	39.2	.....	1.1	.....
12	9.52	9.10	44.8	42.0	1.6	1.45
13	9.88	.....	41.0	.....	10.0	.....
14	7.56	6.72	45.3	43.1	9.6	9.8
15	5.88	.....	47.1	.....	4.0	.....
16	7.84	6.86	44.8	45.9	5.0	4.5
17	5.88	.....	45.3	.....	1.7	.....
18	8.12	7.00	52.3	48.8	2.3	2.0
19	6.16	.....	46.5	.....	1.7	.....
20	7.84	7.00	52.1	49.3	1.3	1.5

In the check pot there is a decided increase in the amount of amino acid nitrogen from the second sampling to the third the results of which are given in table XI. The manured unlimed soils show a slight drop in the amino acid nitrogen, with the exception of the highest manured With the exception of the unmanured soils, all the limed pots show a decided rise, the greatest rise being in the soils with the heaviest application of manure. As before, however, the heavily ma-

TABLE XI

Pot No.	NITROGEN IN P. P. M. AIR DRY SOIL					
	Amino Acid	Ave.	Ammonia	Ave.	Nitrate	Ave.
1	7.56	.....	48.3	.....	24.0	.....
2	7.28	7.42	49.7	49.0	20.8	22.4
3	Lost	.....	44.8	.....	30.0	.....
4	6.16	6.16	46.2	45.5	26.4	28.2
5	6.72	.....	48.3	.....	16.0	.....
6	6.16	6.4	49.7	49.0	14.4	15.2
7	6.72	.....	51.8	.....	11.2	.....
8	7.00	6.86	54.6	53.2	9.6	10.4
9	8.12	.....	54.6	.....	7.6	.....
10	7.84	7.98	56.0	55.3	8.8	8.2
11	9.24	.....	61.6	.....	10.2	.....
12	9.24	9.24	63.0	62.3	12.4	11.3
13	9.24	.....	56.0	.....	10.8	.....
14	9.52	9.38	56.0	56.0	8.8	9.8
15	9.52	.....	51.8	.....	14.0	.....
16	9.52	9.52	54.6	53.2	14.0	14.0
17	10.40	.....	49.7	.....	8.4	.....
18	9.80	1.01	54.6	52.2	5.0	6.7
19	Lost	.....	54.6	.....	4.8	.....
20	11.20	11.2	44.8	49.7	4.0	4.4

nured soils show but slightly higher amounts than the unmanured.

The ammonia results show also an increase over the second sampling. No very consistent differences are to be noted between the limed and the unlimed.

The nitrates are beginning to show quite inconsistent variations. There is, however, a decided increase in all cases. It still holds that increase in manure causes adverse in nitrates.

The results of the fourth sampling are given in table XII. The values for the amino acid nitrogen in the unlimed soils do not appear to be consistent. The check pots, the pots with the highest and lowest amounts of manure, show a slight decrease from the third sampling, while the remainder are somewhat higher. There is no obvious explanation for this. For the limed pots, the amino acid nitrogen shows a decided decrease, the greatest decrease being with the highest amounts of manure.

The values for the ammonia nitrogen also show, in general, a slight decrease, the unlimed slightly greater than the limed. It is quite possible that the lesser decrease in ammonia in the case of the limed pots is bound up with the greater decrease of the amino acid nitrogen in the same pots.

There is a great increase in the amount of nitrates in all cases, the manured pots increasing relatively more than the unmanured. The limed pots, with the higher amounts of manure, show decidedly lower results than the corresponding unlimed soils. Evidently assimilation is still keeping ahead of nitrification.

TABLLE XII

Pot No.	NITROGEN P. P. M. AIR DRY SOIL					
	Amino Acid	Ave.	Ammonia	Ave.	Nitrate	Ave.
1	5.60	.....	45.5	.....	27.2	.....
2	5.32	5.46	44.8	45.2	24.0	25.6
3	5.04	.....	44.1	.....	32.0	.....
4	5.04	5.04	44.8	44.5	35.2	33.6
5	5.04	.....	46.2	.....	28.8	.....
6	5.60	5.32	45.5	45.9	24.0	26.4
7	7.84	.....	45.5	.....	24.8	.....
8	7.84	7.84	49.0	47.3	25.6	25.2
9	7.84	.....	48.3	.....	28.8	.....
10	9.24	8.54	46.9	47.6	33.6	31.2
11	8.12	.....	59.5	.....	40.0	.....
12	9.24	8.68	49.0	54.3	41.6	40.8
13	5.88	.....	51.1	.....	33.6	.....
14	6.72	6.30	66.5	55.8	28.8	31.2
15	Lost	.....	49.0	.....	35.2	.....
16	Lost	Lost	49.0	49.0	28.8	32.0
17	7.28	.....	46.9	.....	19.2	.....
18	Lost	7.28	53.0	49.9	Lost	19.2
19	7.00	.....	51.8	.....	16.0	.....
20	7.56	7.28	46.9	49.3	19.2	17.6

In table XIII, the results obtained on the fifth and last sampling are given. There is, in general, a decrease in the amount of amino acid nitrogen, the greater decrease being, as before, in the limed soils. The more heavily manured soils still show but slightly higher amounts than the unmanured.

The values for the ammonia nitrogen show also a decided decrease from the fourth to the fifth sampling.

There is an increase in the amounts of nitrates in most cases, but the increase is not so great as on the fourth sampling.

TABLE XIII

Pot No.	NITROGEN IN P. P. M. OF AIR DRY SOIL					
	Amino Acid	Ave.	Ammonia	Ave.	Nitrate	Ave.
1	5.60	.....	31.5	.....	35.2	.....
2	4.76	5.18	30.1	3.08	25.6	30.4
2	4.76	5.18	30.1	30.8	25.6	30.4
4	5.04	4.76	31.5	31.5	32.0	30.4
5	6.16	.....	30.1	.....	28.8	.....
6	6.72	6.44	31.5	30.8	32.2	30.5
7	6.44	.....	35.7	.....	20.8	.....
8	6.44	6.44	33.6	34.7	20.8	20.8
9	6.44	.....	39.2	.....	38.4	.....
10	7.00	6.72	30.1	34.7	40.0	39.2
11	8.40	.....	33.6	.....	33.6	.....
12	7.84	8.12	33.6	33.6	44.8	39.2
13	Lost	.....	31.5	.....	32.0	.....
14	3.64	3.64	35.7	33.6	32.0	32.0
15	3.64	.....	37.1	.....	35.2	.....
16	3.78	3.71	33.6	35.4	40.0	37.6
17	3.92	.....	33.6	.....	27.2	.....
18	3.78	3.85	37.1	35.4	Lost	27.2
19	5.32	.....	33.6	.....	36.4	25.8
20	5.32	5.32	41.3	37.5	15.2	.....

The results given in the foregoing tables have been plotted, the points being joined by straight lines. It will not be necessary to discuss the plates much in addition to what has already been written of the tables.

In plate 1, the results for the amino acid nitrogen are plotted. It is seen that in general there is a decrease of these compounds. The values for the manured soils are but slightly higher than for the unmanured. This is perhaps the most striking fact brought out by the experiment. There is no tendency for an accumulation of these products under the conditions of the experiment. Another noteworthy fact emphasized by the curve is the greater consistency of the results for the limed pots. Perhaps a reason for this is that the microbiological activities proceed more normally in a medium of the reaction induced by the lime.

But little more need be said in regard to the ammonia results plotted in plate 2. In a general way, they follow the amino

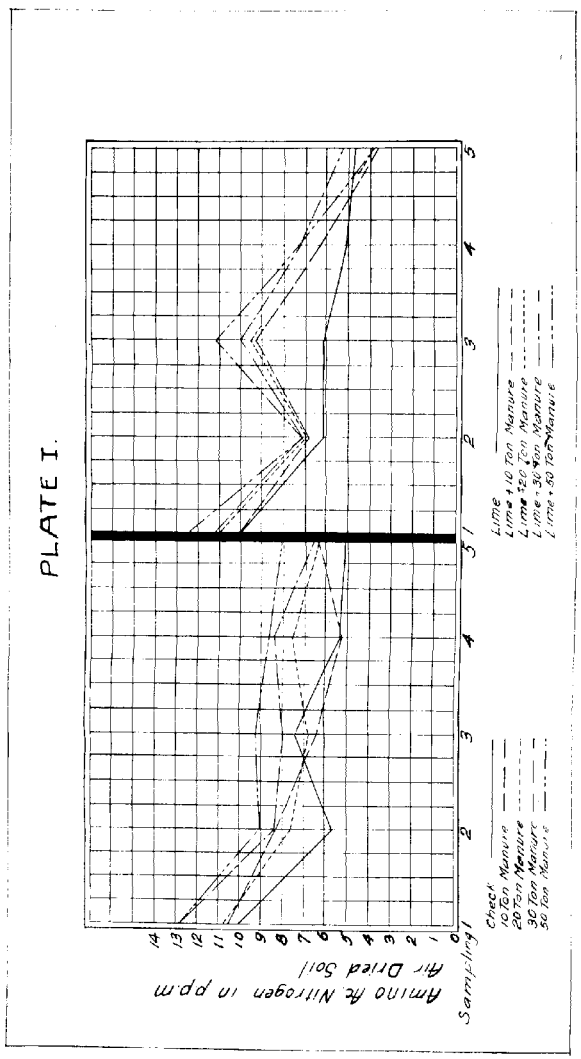
PLATE I.
Amino & Nitrogen in p.p.m
Air Dried Soil
Sampling
Check
10 Ton Manure
20 Ton Manure
30 Ton Manure
90 Ton Manure
Lime
Lime + 10 Ton Manure
Lime + 20 Ton Manure
Lime + 30 Ton Manure
Lime + 90 Ton Manure

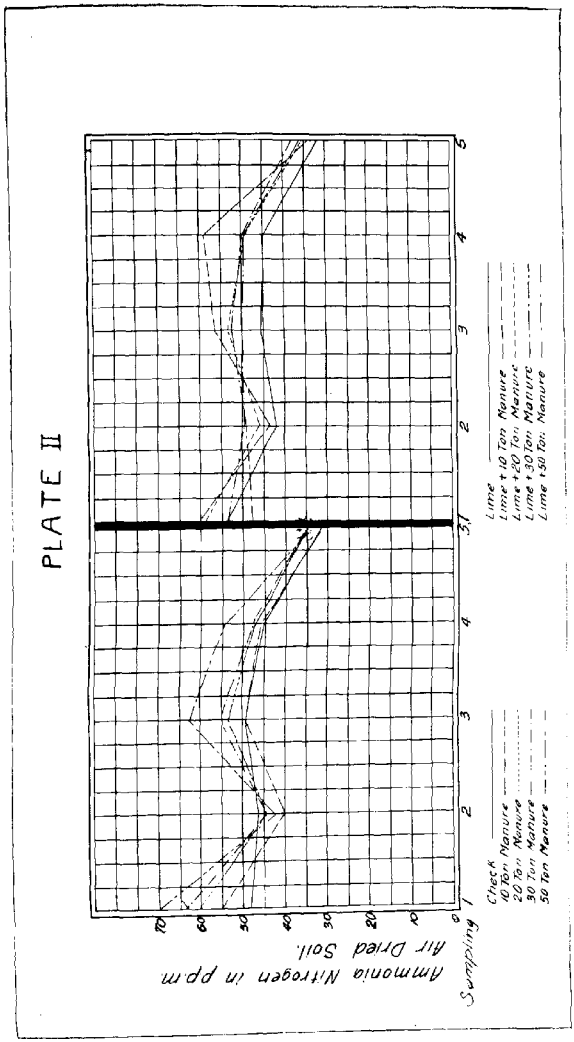
PLATE II
Ammonia Nitrogen in p.p.m.
Air Dried Soil.
Sampling
Check
10 Ton Manure
20 Ton Manure
30 Ton Manure
50 Ton Manure
Lime
Lime + 10 Ton Manure
Lime + 20 Ton Manure
Lime + 30 Ton Manure
Lime + 50 Ton Manure

acid results. When the experiment stopped, as with the amino acid, the ammonia content was decreasing.

In plate 3, which shows the nitrate results, the most striking thing brought out is that the curves for the unmanured soils are below the curves for the manured soils, in the case of the unlimed soils for the first three samplings and for the limed soils for the first four samplings. This points to two conclusions, or a combination of the two, namely denitrification or assimilation, or a combination of the two. If denitrification takes place to any great extent, it might be followed by total nitrogen determinations. In this laboratory at present there are some very carefully controlled experiments along this line being carried out.

Conclusions

The conclusions to be drawn from the work reported in this bulletin are as follows:

1. The work of Chardet, who used the Sorenson method and found from 49 to 68 per cent of the soil nitrogen to be amino acid nitrogen, was not confirmed. Practically no amino acid nitrogen could be found in soil by this method.

2. The Kober method as applied to soils gave the following results:

A. No amino acid nitrogen could be found in the dilute acid extract of soils.

B. Upon adding small quantities of amino acid to a soil and extracting with dilute acids, no amino acid could be found.

C. Upon adding small quantities of amino acids to a soil and extracting with dilute alkali, practically the entire amount added was recovered.

D. There was found to be no difference in the quantity of amino acid nitrogen extracted by dilute alkali in one, two, four and six hours.

D. A few soils were analyzed for free amino acid nitrogen, total amino acid nitrogen and total peptide nitrogen, with the following results:

Soil	Per Cent of Soil Total N. in	NITROGEN IN P. P. M. AIR-DRIED SOIL		
		Total Am. Ac.	*Free Am. Ac.*	*Total Peptide*
A	0.151	7.95	5.45	31.5
B	0.180	23.10	14.70	30.9
C	0.21	23.10	.....	.....

3. The following conclusions may be drawn from the investigations on the nitrate method:

A. Calcium oxide, when shaken with a solution of pure potassium nitrate causes a lowering in the amounts of nitrates found by the phenoldisulphonic acid method in the solution obtained by filtering

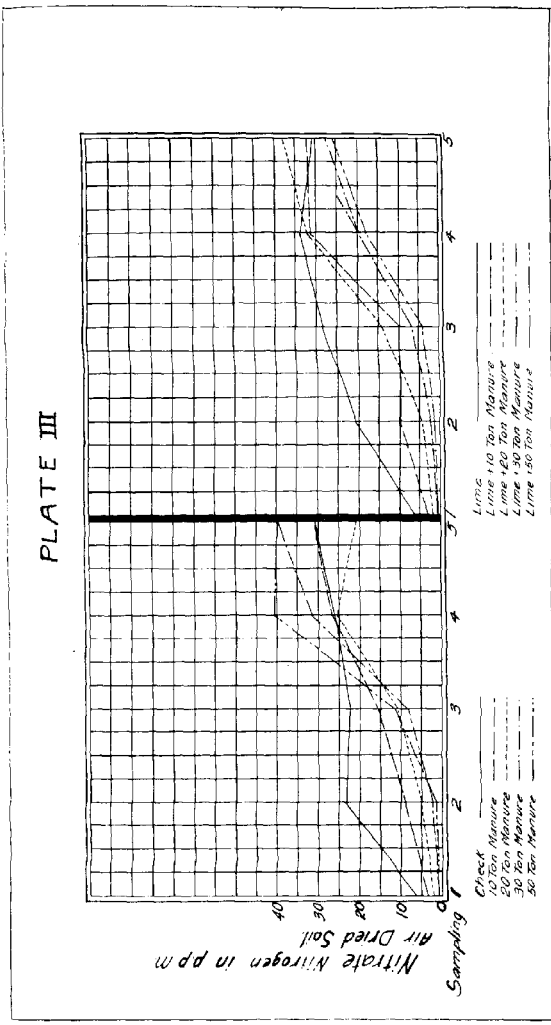
PLATE III
Nitrate Nitrogen in p.p.m
Air Dried Soil
40
30
20
10
0
Sampling
1
2
3
4
5
Check
10 Ton Manure
20 Ton Manure
30 Ton Manure
50 Ton Manure
Lime
Lime +10 Ton Manure
Lime +20 Ton Manure
Lime +30 Ton Manure
Lime +50 Ton Manure

off the excess oxide. Practically the entire amount is given by the aluminum reduction method.

B. Calcium carbonate, used in the place of calcium oxide with pure nitrate solutions causes practically no lowering in the nitrates found by either the phenoldisulphonic acid method or the aluminum reduction method.

C. In soils containing below 20 parts per million of nitrate nitrogen the use of calcium oxide as the flocculating agent gives decidedly lower results and when calcium carbonate is used, the phenoldisulphonic acid method being used in both cases .

D. Upon adding nitrates to soils low in nitrate nitrogen (below 20 p. p. m.) only from 0 to 50 per cent was recovered using calcium oxide as the flocculating agent and the phenoldisulphonic acid method. Substituting calcium carbonate for the oxide about 97 per cent was recovered in most cases.

E. With soils containing between 100 and 200 p. p. m. of nitrate nitrogen per million parts of soil, the use of calcium oxide gives results about 10 per cent lower than when calcium carbonate is used, in both cases the phenoldisulphonic acid method being used. By the aluminum reduction method there is no apparent difference in the action of the two flocculating agents, but the reduction method in all cases gives results decidedly higher than the phenoldisulphonic acid method.

F. The modification of the aluminum reduction method proposed by Burgess was found to be satisfactory in all respects.

The conclusions to be drawn from the pot experiment are as follows:

A. There is no tendency for the amino acid to accumulate under the condition of the experiment, namely in a limed and unlimed acid soil, in a heavily manured and limed, and a heavily manured unlimed acid soil.

B. The amino acid nitrogen was present in the soil in less amounts than the ammonia nitrogen, but in a general way it fluctuates with the ammonia nitrogen.

C. The soils with the higher amounts of manure show a decided decrease in the amount of nitrate nitrogen at first, but after from four to six weeks, there is a decided increase.

July, 1915 Research Bulletin No. 25

BACTERIAL ACTIVITIES AND CROP PRODUCTION

BY P. E. BROWN

AGRICULTURAL EXPERIMENT STATION
IOWA STATE COLLEGE OF AGRICULTURE
AND MECHANIC ARTS

AGRONOMY SECTION
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BACTERIAL ACTIVITIES AND CROP PRODUCTION

By PERCY EDGAR BROWN

Until the present time studies in soil bacteriology have dealt almost exclusively with the occurrence and activities of microorganisms in the soil and no attempt has been made to interpret experimental results from the standpoint of crop production. This is due, of course, to the fact that as a science soil bacteriology is scarcely out of its infancy and the preliminary investigations in any science must of necessity deal mainly with underlying principles and such studies are always apt to be rather fragmentary in character.

A knowledge of the relation of soil bacteria to soil fertility is of importance, however, if the subject is to be of any value in practical agriculture. While, therefore, much work on methods remains to be done, so much knowledge of bacterial action in soils has been accumulated that it seems time now to call attention to the practical phases of the subject; to attempt to correlate the results secured with known facts regarding soil fertility. This need in no way detract from the scientific value of the investigations, but involves merely a broader, more practical interpretation of results.

Many experiments have shown the numbers of bacteria in soils and some of the chemical changes which they bring about. The influence of different soil treatments, both on the numbers of organisms and on the processes which they engender, has also been studied. Only one step further is necessary to show the relation of bacterial numbers and activities to crop production. This step will make soil bacteriology a vital factor which must be considered in ordinary farm practice. It is the step which puts alongside the physical and chemical factors governing soil fertility a third group, the bacteriological, equally important, although largely influenced by the others.

The soil not only furnishes the necessary physical environment for the growth of plants, but it also supplies them with certain chemical compounds which serve as food. This food is not present, however, in a form directly assimilable by plants, but is normally insoluble. Bacteria, of which soils contain vast numbers, are the active agents which bring about the transformation of these insoluble materials into soluble forms. Therefore, it seems probable that a very close relation-

ship exists between such bacterial activities and actual crop production.

Ever since the discovery of the necessity of mineral nutrients in a soluble form for the nourishment of plants attempts have been made to ascertain by chemical means the amount of available plant food in soils with a view of determining their crop-producing power. All these efforts have failed because of the absolute impossibility of imitating bacterial action by means of chemical agents. The importance of bacteriological methods for determining the rate and extent of the production of soluble plant food in a soil, and hence its crop-producing power, is evident therefore.

In most soils nitrogen has been considered the limiting factor of growth and attention has been centered upon investigations dealing with the production of available nitrogen compounds. Nitrates are produced in soils through the action of various groups of bacteria on complex nitrogenous compounds or proteins, normally present. This transformation is accomplished in nature in several stages. One is the production of ammonia and this ammonifying power of a soil, giving as it does an indication of the rate of production of nitrogenous plant food, may be assumed to be closely related to actual crop production. Another stage is the change of ammonia into nitrates, and this nitrifying power of the soil may also be closely allied with its crop producing power. Finally there is the azofying or nitrogen-fixing power of the soil and this may also indicate the amount of crop which may be produced.

THE PURPOSE OF THE EXPERIMENTS

The purpose of the experiments reported in this bulletin was to study certain bacterial activities in field soils in the attempt to secure definite information regarding their relation to the actual crops produced. If special methods of soil treatment exert similar effects on certain bacterial activities and on crops, then it may be assumed that there is a fairly definite relation between the two and the particular bacterial activities in a soil may indicate its crop-producing power. Thus, if the ammonifying power, the nitrifying power, or the azofying power of a soil is enhanced, according to laboratory tests, by some method of soil treatment, and the crop production is also increased, the conclusion that ammonification, nitrification or azofication, and crop production are very closely related would be well warranted. Furthermore, if such results are secured, then it may be assumed that a soil yield-

nitrifying power or an azofying power greater than that of some other soil would be capable of greater crop production, and hence a measure of soil fertility would be secured. The importance of obtaining such a means of determining the crop producing power of soils, can, of course, hardly be estimated.

Experiments covering many years of varying seasons and including tests of all varieties of treatments must, of course, be carried out before any definite conclusions can be reached. The experiments reported in this work indicate that nitrogen transformation in the soil and crop production may be very closely related and they represent, therefore, a preliminary contribution along this line. Some of the results have been reported elsewhere in connection with other studies, but they are assembled here for the purpose of calling especial attention to the relations which they indicate as existing between the bacterial transformations of nitrogen in the soil and the crops produced.

THE FIELD SOILS STUDIED.

Three series of field plots were used, one consisting of 14 one-tenth acre plots located on a uniform soil in the Wisconsin drift soil area and classed by the United States bureau of soils as Carrington loam. The land was somewhat rolling, but not enough to affect seriously its value for experimental purposes. Prior to 1907 it had been under a regular four-year rotation and had been subjected to no special treatment of any kind. In that year the plots were differentiated according to the following plan:

Plot.	Treatment.
601	Continuous corn.
602, 603	2-year rotation, corn and oats.
604, 605, 606	3-year rotation, corn oats and clover.
607, 608	2-year rotation, corn and oats, clover plowed under after the oats.
609, 610	2-year rotation, corn and oats, cowpeas plowed under after the oats.
901, 902	2-year rotation, corn and oats, rye plowed under after the oats.
903	Continuous clover.
904	4-year rotation, corn, corn, oats and clover.

The first tests were carried out in 1911, the fourth year of the special treatment. Plots 601, 602, 604, 607, 609 and 901 were studied during that season, for the reason that they were

treatment could be studied more satisfactorily where the influence of the crop growing at the time of the experiment was presumably the same for all the plots. Plots 903 and 904 were also examined bacteriologically in 1911, but the results are not included here, because of the impossibility of comparing the crop yields. The results from the plots in corn were the only ones considered, because of the impossibility of comparing the bacteriological results from plots under clover and under corn, and also because of the impossibility of comparing the crop yields.*

In 1912 the work begun in 1911 was continued, but in some cases the same plots were not used again for only those in corn were chosen. A plot representing each of the same treatments, however, was investigated. Thus during this season plots 601, 603, 605, 608, 610, 902 and 904 were used. Complete data secured in this work is presented, but the discussion will deal mainly with the relation of the results to the crop yields.

Again in 1913 some bacteriological studies of the soils in this series were carried on, the plots under corn being chosen as previously, plots 601, 602, 606, 607, 609, 901 and 904. Only a comparatively small amount of data was secured, however, owing to the pressure of other work, but the ammonification studies were complete and the results secured may be compared with the crop yields.

The second series of plots studied consisted of five one-tenth acre plots located likewise on the Wisconsin drift soil area and on Carrington loam. These plots were subjected to special treatments of manure in the fall of 1910 as follows:

Plot. No.	Treatment.
1004	Check.
1005	8 tons manure per acre.
1006	12 tons manure per acre.
1007	16 tons manure per acre.
1008	20 tons manure per acre.

*The complete results of the bacteriological studies of these soils in 1911 were reported in Research Bulletin No. 6 of this Station under the title "Bacteriological Studies of Field Soils. II. The Effects of Continuous Cropping and Various Rotations," and the crop yields from the same plots have been reported also. A preliminary statement was made in the bulletin referred to regarding the relation between the bacterial activities studied and the actual crops produced. In the present work, therefore, summaries only of the various bacterial studies which were carried out are given, and special emphasis is placed on the comparison of these results with the crops produced.

Prior to this treatment the soils had been under a regular four-year rotation without any special handling. In 1911 the corn crop suffered very severely from a continued drought, the yields on the manured plots being appreciably less than on the unmanured. Evidently the manure exerted quite a little injury because of the dry weather. In 1912, however, when the experiments reported in the following pages were carried out, the season was favorable and the effects of the manure were evidently more nearly normal.*

The third series of plots was composed of three one-twentieth acre plots located also on the same soil area and consisting of soil of the same type.** Special treatment on these soils consisted in the application of lime as follows:

Plot. No.	Treatment.
510	Check.
509	2 tons ground limestone per acre.
508	3 tons ground limestone per acre.

BACTERIOLOGICAL METHODS

When it became evident that the mere counting of soil organisms could hardly be expected to yield a complete story of the processes occurring in the soil, attention was concentrated on the development of methods for measuring the physiological activities of bacteria. A complete discussion of the results secured by Remy's solution method, which was the first suggested, together with minor modifications of it may be found in another publication.[1] Difficulties in the use of the solution method have been pointed out by the writer [2] and investigations on the use of soil as suggested by Stevens and Withers [3] and Lipman and Brown [4] led to the conclusion that for the examination of field soils, soil itself seemed the most rational medium to employ, offering as it does, conditions as

*The complete results secured from the study of these soils in 1912 have been reported in Research Bulletin No. 13 of this Station under the title, "Bacteriological Studies of Field Soils III. The Effects of Barnyard Manure," and in this work summaries only of the bacteriological results will be given and the emphasis as in the previous cases will be placed upon the comparison of these results with the crop yields.

**A complete study of these plots from the bacteriological standpoint has been reported in Research Bulletin No. 5 of this Station, under the title "Bacteriological Studies of Field Soils. I. The Effects of Lime," and hence merely summaries will be included here for the purpose of correlating the bacterial results with the crop yields.

1. Voorhees and Lipman, Bull. U. S. Dept. Agr. 194. 1907.
2. Brown, P. E., Rsch. Bull. Iowa Agr. Expt. Sta. 11.
3. Science, n. Ser. 27. 704: 991. 1908.
4. New Jersey Agr. Exp. Sta. Report 1908. 129.

closely approximating those in the field as it is possible to secure. The same experiments showed further that fresh soil possessed many advantages over soil in an air-dry condition and its use was recommended.

Recent experiments [5] have suggested that a modified solution method may be of quite as much use as soil itself in testing bacteriological processes, but some of the difficulties attendant upon the use of solutions are not yet obviated. Further improvements are necessary before an artificially prepared solution will represent as satisfactorily as soil itself the physical and chemical conditions in field soils, leaving out of account entirely their bacteriological conditions.

The addition of various materials to soils in laboratory tests to permit of the accumulation of the particular products of bacterial action which it is desired to measure has been studied. Dried blood, cottonseed meal and casein have proven the best for ammonification, dried blood and ammonium sulfate for nitrification, and mannite for azofication. The use of casein for ammonification was suggested in the work of the writer mentioned above and preliminary tests indicated that it would be of value, chiefly because it may be applied to the soil in solution and hence thorough distribution may be accomplished, which is very difficult in the case of the dried blood and cottonseed meal. It is possible, of course, that this material may not always prove satisfactory, but in the experiments reported here no difficulties were encountered and the results are quite satisfactorily comparable with those where dried blood was used.

In the tests of various bacterial activities in field soils reported in this bulletin, several different modifications of the soil method were employed, for the reason that the tests covered a period of several years through which experiments on methods were also being conducted. The results using the different methods are all included, however, as they all tend in the same direction and conclusions are based on a study of the entire mass of data secured.

EXPERIMENTAL

The Rotation Plots. (1911)

In the tests of these soils in 1911, their ammonifying power was determined by the use of dried blood (5 gms.), and of cottonseed meal (5 gms.), in air-dry soil (100 gms.), inoculation being accomplished by adding 20 c. c. of infusions of fresh

5. Lohnis and Green, Centbl. f. Bakt., Abt, II, 40: 457. Green, H. H., Centbl. f. Bakt., Abt. II, 41: 577.

soils. The moisture content was made optimum and the inoculation period was 6 to 7 days at room temperature. Nitrification was tested similarly using dried blood (200 mgs.), and ammonium sulfate (100 mgs.) per 100 grams of soil, inoculating similarly with infusions of fresh soils and incubating for four weeks and azofication was measured by adding mannite (5 gms.) to soil, inoculating as before and incubating for ten days.

Four samplings were made during the season, on June 26, July 8, September 16 and October 25. No samplings were made during August, as a severe drought occurred at that time and it was felt that conditions were too extremely abnormal. The complete data obtained at these various samplings are given in another place [1] and only summarized results are included here.

AMMONIFICATION.

The results of the ammonification tests with dried blood and cottonseed meal are given in tables I and II, respectively. The variations in amount of moisture in the various plots at the same samplings were very small and the differences in bacterial activities which were found could not, therefore, be

TABLE I. THE AMMONIFICATION OF DRIED BLOOD.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
601	171.11	220.74	108.76	110.58
602	178.07	231.38	117.86	116.54
604	188.82	243.60	133.43	131.11
607	175.22	229.63	120.78	124.82
609	179.96	238.93	118.53	116.84
901	174.75	232.08	117.04	114.88

TABLE II. THE AMMONIFICATION OF COTTONSEED MEAL.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
601	142.01	163.32	109.13	111.08
602	144.54	168.74	110.09	122.17
604	151.18	177.81	120.18	126.64
607	145.49	168.71	131.11	123.49
609	148.50	174.00	105.78	119.02
901	144.07	165.94	112.73	115.55

attributed to the different moisture conditions in the plots.

It will be noted that in every case the ammonifying power of the soil under the two-year rotation of corn and oats (602) was greater than that of the soil under continuous corn (601).

1. Rsch. Bull. Iowa Agr. Exp. Sta. 6.

Where the three-year rotation of corn, oats and clover was followed (604), the ammonifying power of the soil was still greater. Where the two-year rotation was modified by the introduction of clover (607), cowpeas (609), or rye (901), as a green manure the ammonifying power of the soil was less than that of the soil under the three-year rotation. Where the clover was used greater ammonification was evidenced in most cases than where the two-year rotation was not so modified. In two instances, at the first two samplings where dried blood was used, the clover did not give an increase in ammonifying power, but the differences in both cases were so slight that they should hardly be considered as placing the general tendency of all the other results in question. In all cases except one the soil under the two-year rotation with clover turned under showed a slightly smaller ammonifying power than under the three-year rotation. The differences in most instances were not large, however.

Where cowpeas were used as the green manure, in every case except two, greater ammonification was found than in soil under the regular two-year rotation, while it was less than that in the soil under the three-year rotation in every instance. The results secured where the cowpeas and the clover were used were so similar that it is impossible to judge definitely of the relative effects of the two; in some cases the clover seemed to give a greater effect on the ammonification and in other instances the cowpeas appeared superior. Conclusions comparing these materials, therefore, cannot be made.

Where the green manure used was rye, in practically every instance the ammonifying power of the soil was less than where the two-year rotation was not so modified. The depressing effect was not pronounced at every sampling, but at least it might be concluded that the rye gave no increase in the ammonifying power of the soil over that occurring where the two-year rotation unmodified was used.

The fact that the green manure crops did not increase the ammonifying power of the soils may have been due to the limitations imposed by the poor moisture conditions in the soil throughout practically the entire season, or possibly to the effect of the organic matter introduced. It is believed that the former explanation is more plausible because the rye crop which was turned under was so much heavier and hence the moisture conditions would be made more unfavorable than where the clover and cowpeas were used.

The ammonification results using dried blood and cottonseed meal did not always run parallel, but the differences were not great and in the majority of cases the same comparisons were secured with the two materials.

TABLE III. THE CROP YIELDS.

Plot No.	Treatment.	Corn. Bushels per Acre.
502	Continuous Corn	35.5
602	Two-year rotation	46.0
604	Three-year rotation	50.7
607	Two-year rotation, clover turned under	52.7
609	Two-year rotation, cowpeas turned under	32.5
901	Two-year rotation, rye turned under	43.2

Comparing now the ammonification results with the crop yields which are given in table III, it will be noted that there is a remarkably good agreement. A greater crop yield was secured where the two-year rotation was followed than on the continuous corn plot, and a still greater yield was secured where the three-year rotation was followed. This corresponds exactly to the ammonification results. Where the clover was introduced into the two-year rotation as a green manure, a greater crop yield was secured than where it was not used. Furthermore a slightly greater yield was obtained than on the three-year rotation plot. This is contrary to the bacteriological results but the difference in yield is hardly large enough to be regarded as appreciable, and it will be recalled that the variations in ammonification, while slightly in favor of the three-year rotation, were not considerable. When cowpeas were used, however, the yield was abnormally depressed, the depression being so great that the yield was less than that on the continuous corn plot. Evidently some unknown factor interfered here, as such a depression is hardly explainable. The ammonification results did not indicate a corresponding depression.

Where the rye was turned under in the two-year rotation the yield was less than on the regular two-year rotation plot and less than on the three-year rotation plot. It was also less than on the plot where clover was used as a green manure. It was greater, however, than where the cowpeas were used but as was mentioned this latter result was undoubtedly abnormal. The ammonification results checked thus in practically every case the crop yields where the rye was used.

The comparison of the crop yields with the ammonification results as a whole shows a surprisingly close relation between the two and leads to the tentative conclusion that the ammonifying power of soils under normal conditions reflects fairly accurately their crop-producing power, and shows quite correctly the relative yields which will be secured.

NITRIFICATION.

In the nitrification tests air-dry soil with dried blood and ammonium sulfate were used as has been described. The

samples were drawn on the same dates as those tested for ammonification and hence the differences in moisture conditions were likewise very slight. The summarized results appear in tables IV and V. The complete data is given in the previous work already mentioned.

The results of the nitrification tests are very largely in accord with those obtained in the ammonification studies. Again, it is noted that the soil under the two-year rotation showed a greater nitrifying power than that under continuous corn, while a still larger nitrifying power was evidenced by the three-year rotation plot. Where clover was introduced into the two-year rotation a gain in nitrifying power was found at the last two samplings, but at the first two dates no increase was evidenced. It will be recalled that a similar situation pertained in the case of ammonification, no gain occurring at the first samplings where clover was used, but at the last two dates considerable increase was found. The nitrifying power of the soil was not increased by the clover in the two-year rotation as much, however, as it was by the use of the three-year rotation.

TABLE IV. THE NITRIFICATION OF DRIED BLOOD.

Plot No.	Mgs. N. I	Mgs. N. II	Mgs. N. III	Mgs. N. IV
601	12.442	19.883	11.864	13.797
602	15.196	23.311	14.620	17.433
604	20.776	27.087	18.173	24.032
607	15.078	22.884	16.410	22.211
609	18.798	25.225	13.453	15.048
901	13.962	20.713	12.711	14.014

TABLE V. THE NITRIFICATION OF AMMONIUM SULFATE.

Plot No.	Mgs. N. I	Mgs. N. II	Mgs. N. III	Mgs. N. IV
601	5.019	17.317	7.565	8.086
602	8.075	24.625	9.788	11.789
604	12.630	24.517	12.903	19.419
607	7.066	21.477	11.357	13.749
609	11.908	22.978	9.101	10.620
901	6.724	21.477	8.319	9.655

When cowpeas were used in the two-year rotation, in most cases there was a slight increase in nitrifying power, but again this increase was less than that brought about under the three-year rotation. Where rye was turned under as a green manure, in every case there was a depression in nitrifying power over that evidenced where the two-year rotation was unmodified. As was suggested in connection with the ammonification results the moisture conditions or the organic matter introduced may have brought about this decrease in

bacterial activities. In all these tests the nitrification of the ammonium sulfate and of the dried blood proceeded almost parallel.

It is evident from the results as a whole that nitrification and ammonification proceeded in the same direction and hence the relations to the crop yields are practically identical. Examining table III again it is seen that increases and decreases in nitrifying power are coincident with increases and decreases in crop yields just as was the case in the ammonification results. It seems, therefore, that the nitrifying power of soils may indicate their crop-producing power, or at least the relative crop-producing power of two soils may be shown.

The close agreement with the ammonification results would indicate further that possibly bacteriological tests of soils for only one of these processes need be carried out in order to judge of the fertility of a soil. Many confirmatory results are, of course, necessary before this latter point can be considered as settled, for it is quite possible that special methods of soil treatment may affect nitrification quite considerably and not influence ammonification and vice versa. Nitrification could hardly be encouraged, however, and ammonification depressed, inasmuch as the latter process precedes the former, but it might be depressed and ammonification increased, in which case there would be an accumulation of ammonium salts in the soil.

AZOFICATION.

The summarized azofication tests are given in table VI, and attention is called merely to the fact that the methods of cropping which affected nitrogen transformations in the soil influenced azofication or nitrogen fixation in the same way. Thus the two-year rotation increased the azofying power over that where corn was grown continuously and the three-year rotation gave a still further increase. When clover was used as a green manure in the two year rotation a gain in azofying power was evidenced except in one instance, but it was less than where the three-year rotation was used. When cowpeas were used there was an increase in azofication over that under the unmodified two-year rotation in only two cases, while in the other two instances no increases occurred. The differences were small, however, and it probably should be concluded that little effect was exerted by the cowpeas. The rye, as in the other bacteriological tests, gave a strong depression in azofying power in every case over that where the regular two-year rotation was used.

These results check almost exactly those secured in the ammonification and nitrification tests, and hence the relations

to the crop yields are just about the same. Referring again to table III, it will be seen that this is the case and increases and decreases in azofying power correspond with similar increases and decreases in crop yields. It is hardly expected, however, that azofication tests can always be relied upon to indicate the crop producing power or fertility of a soil. Conditions which favor nitrification and ammonification need not necessarily favor azofication and hence unless the nitrogen

TABLE VI. THE AZOFICATION TESTS.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
601	9.50	3.93	13.52	10.32
602	17.46	15.07	19.92	17.52
604	20.64	18.25	23.12	20.72
607	14.27	17.46	20.72	18.32
609	18.25	15.87	18.32	16.72
901	14.27	11.88	16.72	15.12

content of the soil is exceedingly small an addition of nitrogen from the atmosphere would probably not be necessary for the production of a large crop yield, the increase in nitrate formation being sufficient to bring about a greater crop growth.

Considering these results as a whole, it is apparent that there is some close relation between crop production and nitrogen transformations in the soil and the possibility of measuring by bacterial tests the fertility of soils is strongly suggested.

THE ROTATION PLOTS. (1912)

The same series of plots was used in 1912 as in 1911, but in some cases different individual plots were employed as again only those cropped to corn were examined. In 1912 601, 603, 605, 608, 610, 902 and 904 were tested, and in 1911, plots 601, 602, 604, 607, 609 and 901. The different rotations studied, with one addition, were however, the same: a two-year rotation of corn and oats; a three-year rotation of corn, oats and clover; a two-year rotation of corn and oats with clover used as a green manure, one with cowpeas as a green manure, and one with rye as a green manure. A four-year rotation plot was also examined in 1912, making one additional plot and constituting the addition to the rotation series mentioned above.

The bacteriological studies during 1912 consisted in an examination of the ammonifying and nitrifying power of the soils at various dates of sampling. The ammonifying power was tested by several different methods; the dried-blood-air-dry-soil method with inoculum from fresh soil was again employed

as in 1911 and the casein-fresh-soil method, and the dried-blood-fresh-soil method were also tested at each date of sampling.

These methods are all described in the bulletin already mentioned [1] and need not be discussed here. Some comparisons are possible from these results, therefore, of the relative values of the different methods. Nitrification tests were carried out by the ammonium-sulfate-air-dry-soil method as it was used in 1911 and by the ammonium-sulfate-fresh-soil method so that a comparison of the air-dry and fresh soil methods is secured.

Azofication tests were not made during this season.

Four samplings were made during the year, on August 9, August 19, October 7 and October 23, the samples being drawn in the usual way with all precautions that they be representative and remain uncontaminated. The moisture content of the soils at the various dates of sampling is given in table VII, and it will be seen that the variations at any one date were so slight that they need not be considered. The crop yields on the plots were secured and the comparisons desired may, therefore, be made of the results of bacterial activities and the actual crops produced.

AMMONIFICATION.

The results of the ammonification tests using dried blood in air-dry soil and inoculating with infusions of fresh soils are given in table VIII in the Appendix, while the summarized results appear in table IX. Examining these results, several

TABLE VII. THE MOISTURE IN SAMPLES.

Plot No.	I Aug. 9 percent	II Aug 19 percent	III Oct. 7 percent	IV Oct. 23 percent
601	20.00	20.00	18.00	17.50
603	17.50	20.00	17.50	18.00
605	17.50	20.00	20.00	19.00
608	17.50	19.50	20.00	18.50
610	17.50	20.00	18.00	17.00
902	17.50	18.50	16.00	15.50
904	14.00	18.00	15.00	15.00

TABLE IX. AMMONIFICATION OF DRIED BLOOD IN AIR DRY SOIL.

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
601	148.33	54.93	124.78	122.42
603	157.55	66.51	130.27	127.92
605	170.69	79.77	138.71	138.71
608	179.65	82.40	141.85	143.42
610	168.53	75.73	136.95	130.67
902	151.27	64.15	125.17	119.09
904	161.08	71.61	131.06	128.21

1. Rsch. Bull. Iowa Agr. Exp. Sta. 11.

facts appear quite distinctly. In every case the two-year rotation of corn and oats (603) showed a greater ammonifying power than the plot under continuous corn (601), and three-year rotation of corn, oats, and clover (605) gave a still greater ammonification. The greatest ammonification in any of the soils was given by the two-year rotation plot where clover was turned under (608). Where the green manure crop in the two-year rotation was cowpeas (610), a smaller ammonifying power was shown than where the three-year rotation was followed, but it was greater than where the two-year rotation was not modified. Where rye was used as the green manure crop in the two-year rotation (902) a pronounced depression in ammonification over that shown where the rye was not used was shown. In only one instance, however, did the ammonifying power fall below that of the continuous corn plot. Where the four-year rotation of corn, corn, oats and clover was used (904), the ammonifying power of the soil was slightly less than that in the three-year rotation plot, but is was greater than in the two-year rotation plot.

The ammonification results using the dried-blood-fresh-soil method appear in table X in the appendix, and the summarized results in table XI. The lower amounts of ammonia produced in this case are due to the shorter period of incubation which was practised here, for by this method the incubation period was five days, while in the previous determinations it was seven days. It will be unnecessary to discuss these results in detail as there is practically absolute agreement with the results secured where the dried-blood-air-dry-soil method was employed. The differences are brought out even more distinctly in these tests and the rank of the soils in ammonifying power is the same.

TABLE XI.
AMMONIFICATION OF DRIED BLOOD IN FRESH SOIL.

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
601	106.34	68.66	50.81	54.74
603	110.66	80.05	65.14	62.39
605	117.32	85.70	73.77	71.02
608	120.87	88.28	74.02	74.66
610	115.95	78.87	72.59	71.41
902	109.67	73.38	58.86	62.19
904	114.14	82.90	68.28	69.17

The ammonification results, using the casein-fresh-soil method, are given in table XII, in the appendix, and the summarized results in table XIII. The amounts of ammonia produced here are much smaller than where the dried blood

methods were used and hence the difficulty in distillation is very much less. Again there is practically uniform agreement with the previous results and these figures will not be discussed in detail. In practically every case the soils stood in the same relationship to each other in ammonifying power as they did where the dried blood was employed.

It is apparent from these results that the ammonification tests with these particular field soils gave practically identical results whether dried blood was used in air-dry inoculated soil or in fresh soil or whether casein was used in fresh soil. There is much greater difficulty in securing the agreement of duplicate determinations where dried blood is employed and it is particularly difficult to mix that material thoroughly with fresh soil samples. Smaller amounts of ammonia were produced where the casein was used, due, of course, mainly to the shorter period of incubation, and the agreement of duplicates was very satisfactory. These results check the previous experiments in indicating that the casein-fresh-soil method may prove of value in ammonification studies.

TABLE XIII. AMMONIFICATION OF CASEIN.

Plot No.	I Ammonia Mgs. N.	II Ammonia Mgs. N.	III Ammonia Mgs. N.	IV Ammonia Mgs. N.
601	61.80	64.84	58.66	55.33
603	67.30	71.80	65.13	66.31
605	71.61	76.52	68.47	69.45
608	72.30	79.07	70.63	72.70
610	68.67	73.87	67.20	69.25
902	62.78	69.06	63.18	62.39
904	67.10	73.37	67.10	68.67

TABLE XIV. THE CROP YIELDS.

Plot No.	Treatment.	Yield per acre (1912)
601	Continuous Corn.	50.25 bu. Corn
603	Corn & Oats	63.12 bu. "
605	Corn, Oats & Clover	69.00 bu. "
608	Corn & Oats, Clover turned over	74.00 bu. "
610	Corn & Oats, Cowpeas turned under	68.50 bu. "
902	Corn & Oats, Rye turned under	59.50 bu. "
904	Corn, Corn, Oats & Clover	67.50 bu. Corn

Comparing now the results of the ammonification studies with the crop yields which appear in table XIV quite striking comparisons are noted. The order of the plots in ammonifying power in all the tests was:

608—Two year rotation—clover turned under.
605—Three year rotation.
610—Two year rotation—cowpeas turned under.
904—Four year rotation.
603—Two year rotation.
902—Two year rotation—rye turned under.
601—Continuous corn.

It will be seen that arranging the plots according to crop yields for this season they would stand in identically the same order. It would seem, therefore, that there must be some close relationship between the ammonifying power of soils and their crop production.

NITRIFICATION.

In the nitrification tests carried out in 1912 the ammonium-sulfate-air-dry soil method and the ammonium-sulfate-fresh-soil method were used. The results with the former method are given in table XV in the appendix, and the summarized results in table XVI. Examining this latter table, it is found that the nitrifying power of the soils apparently varied considerably where different methods of treatment were employed. The continuous corn plot showed the smallest nitrifying power just as it did the smallest ammonifying power. The three-year rotation plot gave a greater nitrification than the two-year rotation plot, while the two-year rotation plot where clover was turned under showed a still greater nitrification. The two-year rotation plot where cowpeas were used as a green manure showed a greater nitrifying power than the unmodified two-year rotation soil, but it was less than that of the soil under the three-year rotation. When rye was used in the two-year rotation

TABLE XVI. NITRIFICATION OF AMMONIUM SULFATE IN AIR DRY SOIL.

Plot No.	I Nitrates Mgs. N.	II Nitrates Mgs. N.	III Nitrates Mgs. N.	IV. Nitrates Mgs. N.
601	10.431	12.443	8.444	7.232
603	13.489	16.751	12.427	11.333
605	15.114	18.941	15.546	14.557
608	15.250	23.931	16.524	15.250
610	14.196	18.110	15.208	14.733
902	12.695	12.893	9.914	10.936
904	14.434	17.410	14.946	14.686

TABLE XVIII. NITRIFICATION OF AMMONIUM SULFATE IN FRESH SOIL.

Plot No.	I. Nitrates Mgs. N.	II. Nitrates Mgs. N.	III. Nitrates Mgs. N.	IV. Nitrates Mgs. N.
601	11.944	15.300	7.183	6.844
603	12.728	16.601	10.695	9.776
605	14.682	22.583	12.462	12.154
608	15.520	25.078	13.784	14.224
610	13.559	18.264	12.233	13.999
902	11.960	15.837	7.789	10.629
904	13.060	17.414	10.981	13.166

just as was noted in the ammonifying studies the nitrifying power was depressed below that of the unmodified two-year rotation soil. The four-year rotation gave a nitrifying power

greater than the two-year rotation but less than the three-year rotation and less than the two-year rotation with clover or cowpeas turned under for green manure.

The results of the nitrification tests where the ammonium-sulfate-fresh-soil method was used appear in table XVII in the appendix and the summarized results in table XVIII. It will be unnecessary to discuss these results as they check practically identically the tests with the other method. The soils rank exactly the same in nitrifying power by both methods.

The results of the nitrification tests and the crop yields and the ammonification studies agree exactly. The ranking of the soils in crop yields, in ammonification and in nitrification is identical.

It is evident, therefore, that the ammonification and nitrification of nitrogenous organic material in soils and their crop-producing powers are very closely related and that tests of the power of soils to produce ammonia or nitrates may be an indication of their crop-producing power, or at least of their relative crop-producing ability.

These results also confirm previous observations, that the ammonifying power of soils and their nitrifying powers may be similarly effected such of course may not always be the case as it is possible to conceive of conditions affecting the nitrifying organisms which will not similarly influence the ammonifiers. The latter include a large class of organisms of varying characteristics and requirements for optimum growth while the former include only a comparatively small number of organisms similarly affected by surrounding conditions. Under ordinary conditions in field soils, however, these results in corroboration of others previously secured indicate that soil conditions favoring the one process apparently similarly favor the other. It would seem, therefore, that only the ammonifying power of soils, or their nitrifying power, need be determined.

The Rotation Plots, 1913

The experiments were continued in 1913 on the same series of plots as in 1912, using again only those which were in corn, plots 601, 602, 606, 607, 609, 901 and 904. Ammonification results only were secured as the tests the previous year had indicated that there was practically no difference between the results of ammonification and nitrification tests. The casein-fresh-soil method was the only one employed here partly because of the pressure of the other work and partly because the other methods previously employed had shown similar results to those secured with the casein method. Three samplings were made, on August 15, August 23 and August

26. The moisture content of the soils at the different samplings is given in table XIX, and it is seen that the variations were so small that they may be considered negligible. The crop yields were secured as previously.

TABLE XIX. MOISTURE IN SAMPLES.

Plot No.	I Aug. 15 percent	II Aug. 23 percent	III Aug. 26 percent
601	13.50	17.50	15.00
602	13.50	17.50	14.00
606	14.00	15.00	14.00
607	14.50	11.00	15.00
609	10.00	12.50	13.00
901	12.50	12.50	13.50
904	12.00	11.00	14.00

The ammonification results are given in table XX, in the appendix and are summarized in table XXI. Again the soil under continuous corn showed the smallest ammonification, that under the two-year rotation gave a somewhat greater ammonifying power, and that under the three-year rotation showed a still greater power. In this case, however, the plot under the two-year rotation with clover turned under showed a smaller ammonification than under the three-year rotation, while the reverse was true in 1912. The ammonification was greater, however, than in the two-year rotation plot unmodified.

TABLE XXI. THE AMMONIFICATION OF CASEIN (1913).

Plot No.	August 15 Mgs. N.	August 23 Mgs. N.	August 26 Mgs. N.
601	68.38	60.82	55.67
602	71.56	63.47	59.31
606	78.74	69.59	64.05
607	74.89	66.35	63.15
609	73.53	64.45	60.52
901	75.65	68.15	63.46
904	74.28	65.21	60.97

TABLE XXII. CROP YIELDS 1913.

Plot No.	Treatment	Yield per acre
601	Continuous corn.	30.0 bu.
602	2 yr. rotation, corn and oats	53.3 "
606	3 yr. rotation, corn, oats and clover	68.0 "
607	2 yr. rotation, corn and oats, clover turned under	64.0 "
609	2 yr. rotation, corn & oats, cowpeas turned under	60.0 "
901	2 yr. rotation, corn and oats, rye turned under	65.3 "
904	4 yr. rotation, corn, corn, oats and clover	62.6 "

The soil under the two-year rotation where cowpeas was used as the green manure showed a slightly smaller ammonifying power than that under the two-year rotation where clover was used, but it was likewise greater than that shown by the soil where the two-year rotation was not modified. During this season the rye turned under in the two-year rotation did not depress the ammonifying power as it did in the two previous years. This soil actually showed a greater ammonifying power than those where the clover or the cowpeas were used. It was less, however, than that in the three-year rotation plot. The four-year rotation plot showed a smaller ammonification than the three-year rotation plot or the two-year rotation plot where clover or where rye was used, but it was greater than that in the two-year rotation plot or that where cowpeas were used.

Comparing these results with the crop yields given in table XXII, it is apparent that the indications of fertility given by the ammonification studies are borne out by the actual crops secured. The rank of the soils, both in ammonifying power and in crop production, was thus:

606—Three year rotation.
901—Two year rotation—rye turned under.
607—Two year rotation—clover turned under.
904—Four year rotation.
609—Two year rotation—cowpeas turned under.
602—Two year rotation.
601—continuous corn.

The results of these studies check those of previous years, therefore, and indicate that ammonification and crop production are very closely related and the determinations of the ammonifying power of the soil made during the growing season may show the relative crop producing powers of the soils. The actual amounts of crops produced at the harvest were in complete agreement with the relative amounts indicated by the ammonifying powers of the soils.

The Manured Plots, 1912

The manured plots tested in 1912, consisted of five plots, four of which were treated with various amounts of barnyard manure. The bacteriological tests consisted in studies of the ammonifying powers and nitrifying powers of the soils by the various methods which have been described. The crop yields on the plots were secured and hence a comparison of the tests of bacterial activities and crop production is possible also on these soils. The complete results of these tests having already been published, summarized tables will only be given here.

There are included the results of the ammonification studies, using the casein-fresh-soil, the dried-blood-air-dry-soil, and the dried-blood-fresh-soil methods, and the results of the nitrification tests using the ammonium-sulfate-air-dry-soil, and the ammonium-sulfate-fresh-soil methods. Four samplings were made of these soils during the season of the experiment on August 2, August 15, August 22 and September 9. The moisture content of the soils varied slightly at the different samplings, but there was very slight difference in the amount of water in the different plots at the same dates and hence the differences in ammonification and nitrification must be attributed to the different treatments rather than to any variations in moisture content.

The samples were drawn as usual with all precautions to avoid any contamination and in the manner which has already been described.

AMMONIFICATION.

The results of the ammonification tests appear in tables XXIII, XXIV and XXV. Table XXIII, which gives the results using the casein-fresh-soil method, shows that the soil treated with eight tons of manure per acre (1005) gave a greater ammonifying power than the untreated soil (1004); the soil receiving 12 tons of manure (1006) showed a higher ammonifying power than that receiving the smaller amount of manure, and the soil where 16 tons of manure per acre were used (1007) gave a still higher ammonification. When 20 tons of manure were applied, however, (1008) the ammonification was less than that where the 12 and 16 ton amounts were employed but still greater than that where the eight ton amount was used. Evidently the 20 tons of manure depressed ammonification.

TABLE XXIII. THE AMMONIFICATION OF CASEIN.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	37.87	68.27	67.49	51.60
1005	46.89	73.57	72.79	58.86
1006	51.79	77.50	78.87	66.32
1007	51.99	78.48	79.46	65.72
1008	48.78	75.14	74.75	60.42

TABLE XXIV. THE AMMONIFICATION OF DRIED BLOOD. (FRESH SOIL).

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	66.90	83.97	73.57	66.71
1005	84.76	92.21	83.97	70.63
1006	86.32	106.34	98.88	85.54
1007	97.90	109.47	98.88	84.95
1008	86.72	95.74	87.50	76.91

TABLE XXV. THE AMMONIFICATION OF DRIED BLOOD. (AIR-DRY SOIL).

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	80.44	111.83	106.34	102.81
1005	94.76	117.33	109.47	117.13
1006	100.06	131.25	122.23	127.92
1007	100.85	137.14	124.00	133.02
1008	95.75	128.90	113.80	122.52

Comparing these results with the crop yields given in table XXVI, it will be found that the same effects of the manure were evidenced on the crop as were shown on the ammonification. The 20 ton amount of manure depressed the yield of corn, below that secured where the 12 and 16 ton amounts were used.

Examining the results secured in the ammonification tests where the dried blood methods were used it is seen that the effects of the manure on the ammonifying power of the soil were exactly the same as were shown by the casein method. An increase in ammonification was found when manure was applied up to the 16 ton amount and beyond that point a depression occurred.

The amounts of ammonia secured where the air-dry soil was used were considerably larger than where the fresh soil was employed. This was due as in previous experiments to the different period of incubation employed. The fresh soil tests were incubated for five days while the air-dry soil tests were incubated for seven days.

The comparisons were the same, however, in both cases. It is evident from these tests that the ammonification of casein in fresh soil and of dried blood in fresh soil or in air-dry soil inoculated with infusions of fresh soils proceeded in the same direction and the same differences in the ammonifying powers of soils differently treated were indicated by any of the methods.

TABLE XXVI. THE CROP YIELDS.

Plot No.	Treatment	Corn, (1912)
1004	Check	50.50 Bu.
1005	8 T. Manure	77.62 Bu.
1006	12 T. Manure	86.00 Bu.
1007	16 T. Manure	87.00 Bu.
1008	20 T. Manure	84.00 Bu.

Again it is shown that the ammonifying power of the soils according to any of the methods used indicated quite accurately their relative crop-producing power.

NITRIFICATION.

The results of the nitrification tests using the ammonium-sulfate-air-dry-soil method are given in table XXVII; those secured where the fresh soil was employed are shown in table XXVIII. It will be unnecessary to discuss these results individually as the relations between the nitrifying powers of the different soils were the same in both cases. As has been noted previously similar results were secured in nitrification tests whether the air-dry soil method or the fresh soil method was used.

Just as was noted in ammonification, applications of manure in gradually increasing amounts, up to the 16 ton application, increased the nitrifying power of the soil. Thus the nitrifying power of the soil in plot 1005 was greater than that in 1004, that in 1006 was still greater and in practically every case that in 1007 was larger than that in 1006. When the application of manure was increased beyond 16 tons, however, a depression in nitrification occurred. Thus the nitrifying power of soil 1008 was less than that of 1006 and 1007 where the 12 and 16 ton amounts were used, but it was greater than that of the soil receiving the eight ton application.

TABLE XXVII. THE NITRIFICATION OF $(NH_4)_2\ SO_4$

(Air-Dry Soil).

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	8.507	14.794	12.506	9.211
1005	9.326	15.153	13.693	10.262
1006	10.000	17.716	14.792	12.593
1007	11.655	18.712	16.401	12.446
1008	10.964	16.696	14.662	10.444

TABLE XXVIII. THE NITRIFICATION OF $(NH_4)_2\ SO_4$
(Fresh Soil).

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
1004	5.576	10.946	10.283	9.141
1005	7.259	12.583	12.543	10.000
1006	8.470	16.733	14.142	12.698
1007	10.282	18.694	15.641	13.011
1008	8.125	16.164	12.949	10.528

Comparison of these results with the crop yields and with the ammonification results, shows exact agreement. Applications of manure up to 16 tons per acre increased the ammonifying power and the nitrifying power of the soil and the crop yield to the same extent. These results check the previous observations that ammonification and nitrification tests may often run parallel. In this case, as in the studies of the rota-

tion plots, the treatment which affected ammonification in a certain way influenced nitrification similarly.

Previous results are also confirmed regarding the relation between crop yields and certain bacterial activities. Tests of the ammonifying power of soils or of their nitrifying powers apparently indicate quite accurately their crop producing powers. There must, therefore, be some close relation between the transformation of nitrogenous material into ammonia and nitrates and the ability of the soil to produce crops.

The Limed Plots. (1911)

Three plots were studied in this series of experiments, two of which received applications of limestone at the rate of two and three tons per acre.

The bacteriological tests included studies of ammonification by the dried blood and cottonseed meal methods, tests of nitrification by the ammonium sulfate and dried blood methods, and of azofication by the mannite method. In all these methods air-dry soil was used and an inoculum of infusions of fresh soil added in every case. These methods are the same as those used in the rotation plot studies in the same year. The crop yields were secured as usual and comparisons of these with the results of bacterial activities are, therefore, possible.

Four samplings were made from these plots during the season, on June 21, July 6, September 14 and October 24. The samples were drawn as described in the bulletin already mentioned. The moisture content of the soils is given in table XXIX, which shows the check plot (510) contained more moisture at every date of sampling than the other two; plot 508, receiving the three tons of ground limestone, had the smallest moisture content. This fact is important in explaining the lack of any more pronounced gain in crop yield in that plot over the untreated plot and over that receiving the two ton application.

AMMONIFICATION.

The ammonification of dried blood and of cottonseed meal is shown in tables XXX and XXXI. It is apparent that the application of limestone increased the ammonifying power when tested either by the use of dried blood or of cottonseed meal. Furthermore the three ton application of lime gave a greater increase than the two ton amount in every case, although the greatest increase occurred between the untreated soil and that receiving the two ton application.

TABLE XXIX. MOISTURE IN SAMPLES.

Plot No.	I June 21 Percent.	II July 6 Percent.	III Sept. 14 Percent.	IV Oct. 24 Percent.
510	20.0	15.0	20.0	18.75
509	19.0	12.5	19.5	17.00
508	17.0	11.0	19.5	17.00

TABLE XXX. THE AMMONIFICATION OF DRIED BLOOD.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
510	207.17	206.60	128.06	129.78
509	208.12	207.30	144.51	140.05
508	214.13	235.22	155.59	149.32

TABLE XXXI.
THE AMMONIFICATION OF COTTONSEED MEAL.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
510	131.26	157.22	126.22	124.32
509	132.68	161.06	141.15	130.28
508	142.01	172.58	151.22	137.90

Comparison of these results with the crop yields given in table XXXII shows very good agreement, altho the effect of the lime on the crop yield is not very great. It will be recalled that the moisture content of the limed plots was less than that of the check plot in every case and it appears that this difference in water content prevented any more definite increase in crop. The increases in ammonification due to lime were evidently much less than they should have been had the plots been more uniform in this particular. The data, however, serve to show an agreement between the ammonification tests and the crop yields which check the previous observations along the same line.

NITRIFICATION.

The results of the nitrification tests are given in tables XXXIII and XXXIV, the former showing the tests with dried blood and the latter with ammonium sulfate. The applications of ground limestone increased considerably the nitrifying power of the soil, the three ton amount giving a larger increase than the two ton amount. This was true at every date of sampling and in both series of tests. Evidently the tests for nitrification with dried blood and with ammonium sulfate indicate similar differences in soils differently treated.

Comparing these results with the crop yields and with the ammonification tests there is found to be exact agreement. The nitrification and ammonification tests in this experiment

just as has been observed previously run parallel and either therefore may indicate the crop-producing power of the soil.

TABLE XXXII. THE CROP YIELDS.

Plot No.	Treatment	Corn Bu.
510	Check	52.5
509	2 T. Lime	55.0
508	3 T. Lime	55.0

TABLE XXXIII. THE NITRIFICATION OF DRIED BLOOD.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
510	13.745	27.056	20.579	14.570
509	15.844	33.857	23.247	18.434
508	21.911	39.686	29.376	22.946

TABLE XXXIV. THE NITRIFICATION OF AMMONIUM SULFATE.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
510	8.737	24.987	14.208	8.762
509	10.547	25.475	20.146	11.743
508	14.822	29.034	24.061	17.890

AZOFICATION.

The azofication tests were carried out as usual and the results are given in table XXXV. It is apparent here that lime increased considerably the azofying power of the soil and the three ton application gave a greater increase than the two ton amount.

Comparing these results with those of the previous bacteriological studies, it is found that there is complete agreement. There is also agreement with the crop yields, although these, as has been mentioned, are not so distinctive due evidently to the differences in moisture content of the plots which were more pronounced during the latter part of the season.

As a whole, however, these studies check remarkably well the experiments on plots under other methods of treatment and show that bacterial transformations of nitrogenous compounds in the soil or rather the ability of soils to bring about the simplification of nitrogenous materials or the addition of nitrogen may be considerably modified by various methods of soil treatment. Furthermore, they check previous results in showing that certain bacterial activities in the soil may be very closely related to the actual crop-producing power of the soil. The ammonifying power of soils, their nitrifying power or even their azofying power may, therefore,

indicate their crop producing power, or at least the relative crop producing power of several soils.

TABLE XXXV. THE AZOFICATION TESTS.

Plot No.	I Mgs. N.	II Mgs. N.	III Mgs. N.	IV Mgs. N.
510	5.52	2.34	11.89	11.09
509	15.07	16.66	25.41	27.00
508	26.21	30.19	38.93	37.34

CONCLUSIONS

These experiments as a whole represent a line of investigations in soil bacteriology which it is believed will ultimately place the subject on a more practical basis, a basis which will permit of direct application of results secured to the solution of soil fertility problems. It was purposed at the commencement of the work to continue the study through a long period of years before attempting to draw any conclusions, but unforseen circumstances demanded the relinquishing of the plots which formed the basis of this work, and hence the tests planned must be discontinued for the present. A new series of plots has been laid out on a new farm, and this work will be continued on these new plots as soon as they have been under special treatment for a long enough period to permit of their satisfactory differentiation.

Furthermore, in this work the relations between the bacterial activities studied and the actual crop yields on these plots have proven so striking and so consistent that it was felt that accidental coincidence had been practically eliminated, and that the results might be considered to give a strong indication that certain bacterial activities in field soils are very closely associated with crop yields.

The work reported in this bulletin, therefore, while of course far from conclusive, permits of the tentative conclusion that bacterial activities involved in the transformation of nitrogenous organic matter in the soil bear a very close relationship to the actual crop yields secured on the same soils.

Furthermore, the results indicate that laboratory tests of such bacterial activities may indicate quite accurately the crop-producing power of a soil, or at least the relative crop-producing power of several soils.

If further tests confirm these conclusions, it may be possible in the more or less distant future to outline definite laboratory tests for the determination of the relative fertility or crop-producing power of soils.

TABLE VIII. THE AMMONIFICATION OF DRIED BLOOD IN AIR-DRY SOIL.

I				II			III			IV		
Plot No.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.
601	201	150.29		701	58.47		6001	123.21		6129	124.78	
	202	146.37	148.33	702	51.40	54.93	6002	126.35	124.78	6130	120.07	122.42
603	205	158.14		705	62.78		6003	132.24		6131	124.78	
	206	156.96	157 55	706	69.85	66.31	6004	128.31	130.27	6132	131.06	127.92
605	209	175.80		709	78.09		6005	136.56		6133	133.42	
	210	165.59	170.69	710	80.44	79.77	6006	140.87	138 71	6134	144.01	138.71
608	215	176 58		715	85.54		6007	133.02		6135	134.99	
	216	168.73	172.65	716	79.26	82.40	6008	150.68	141.85	6136	151.86	143.42
610	219	167.95		719	78.09		6009	131.85		6137	133.42	
	220	169.12	168.53	720	73.38	75.73	6010	142.05	136.95	6138	127.92	130.67
902	223	146.37		723	65.53		6011	129 49		6139	118.50	
	224	156.18	151.27	724	62.78	64.15	6012	120.86	125.17	6140	119.68	119 09
904	227	165.20		727	68.67		6013	133.02		6143	135.77	
	228	156.96	161.08	728	74.56	71.61	6016	139.10	131.06	6144	141.66	138.21

TABLE X. THE AMMONIFICATION OF DRIED BLOOD IN FRESH SOIL.

601	229	108.30		729	69.45		6017	52.19		6145	56.51	
	230	104 38	106.34	730	67.88	68.66	6018	49.44	50 81	6146	52.97	54.74
603	233	109.09		733	78.09		6019	64.75		6147	60.43	
	234	112.22	110,66	734	82.01	80.05	6020	65.53	65.14	6148	64.35	62.39
605	237	118.50		737	86.70		6021	74 16		6149	67.49	
	238	116.15	117.32	738	86.70	86.70	6022	73.38	73.77	6150	74.56	71.02
608	243	124.80		743	89 86		6023	74.06		6151	76.73	
	244	116.94	120.87	744	86.70	88.28	6024	73.38	74.02	6152	72.59	74.66
610	247	117.72		747	82.01		6025	72.99		6153	69.45	
	248	114.18	115.95	748	75.73	78.87	6026	72.20	72.59	6154	73.38	71.41
902	251	108.30		751	76.52		6027	57.29		6155	62.78	
	252	111.05	109 67	752	70.24	73.38	6028	60.43	58.86	6156	61.61	62.19
904	255	115.76		755	83.97		6031	69.85		6159	71.63	
	256	112.53	114 14	756	81.83	82.90	6032	66.71	68.28	6160	66.71	69.17

TABLE XII. THE AMMONIFICATION OF CASEIN.

I				II			III		IV		
Plot No.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.
601	257	61.61		757	65.33		6033	58.47		6161	55.72	
	258	62.00	61.80	758	64.35	64.84	6034	59.86	58.66	6162	54.94	55.33
603	261	67.89		761	71.02		6035	64.35		6163	55.71	
	262	66.71	67.30	762	72.59	71.80	6036	65.92	65.13	6164	65.92	66.31
605	265	70.63		765	78.09		6037	69.06		6165	68.28	
	266	72.59	71.61	766	74.95	76.52	6038	67.89	68.47	3166	70.63	69.45
608	271	70.63		771	79.52		6039	70.63		6167	72.99	
	272	74.16	72.39	772	81.62	79.07	6040	70.63	70.63	6168	72.59	72.79
610	275	67.49		775	72.59		6041	66.71		6169	69.06	
	276	69.85	68.67	776	74.16	73.37	6042	67.98	67.49	6170	69.45	69.25
902	279	62.72		779	69.85		6043	61.11		6171	62.00	
	280	62.78	62.78	780	68.27	69.06	6044	64.75	63.18	6172	62.78	62.39
901	283	66.71		783	74.16		6047	67.10		6175	68.28	
	284	67.49	67.10	784	72.59	73.37	6048	67.10	67.10	6176	69.06	68.67

TABLE XV. THE NITRIFICATION OF AMMONIUM SULPHATE IN AIR-DRY SOIL.

I				II			III			IV		
Plot No.	Lab. No.	Nitrate Mgs. N.	Av. Mgs. N.	Lab. No.	Nitrate Mgs. N.	Av. Mgs. N.	Lab. No.	Nitrate Mgs. N.	Av. Mgs. N.	Lab. No.	Nitrate Mgs. N.	Av. Mgs. N.
601	369	10.862		869	12.500		6097	8.000		6225	7.465	
	370	10.000	10.431	870	12.387	12.443	6098	8.888	8.444	6226	7.000	7.232
603	373	13.682		873	16.665		6099	12.355		6227	11.000	
	374	13.296	13.489	874	16.837	16.751	6100	12.500	12.427	6229	11.667	11.333
605	377	15.000		877	19.000		6101	15.097		6230	14.739	
	378	15.228	15.114	878	18.882	18.941	6102	15.425	15.546	6231	14.675	14.557
608	383	15.000		883	23.862		6103	16.605		6232	15.000	
	384	15.500	15.250	884	24.000	23.931	6104	16.384	16.524	6233	15.000	15.250
610	387	14.282		887	18.328		6105	15.129		6234	14.466	
	388	14.111	14.196	888	17.892	18.110	6106	15.287	15.208	6235	11.000	14.733
902	391	12.822		891	12.862		6107	10.000		6236	10.872	
	392	12.568	12.695	892	12.925	12.893	6108	9.829	9.914	6259	15.000	10.936
904	395	14.500		895	17.286		6111	14.892		6240	14.372	
	396	14.369	14.434	896	17.534	17.410	6112	15.000	14.946			14.686

TABLE XVII. THE NITRIFICATION OF AMMONIUM SULPHATE IN FRESH SOIL.

601	397	12.000		897	15.600		6113	7.000		6241	6.889	
	398	11.888	11.944	898	15.000	15.300	6114	7.366	7.183	6242	6.800	6.844
603	401	12.568		901	16.665		6115	10.522		6243	9.085	
	402	12.889	12.728	902	16.537	16.601	6116	10.868	10.695	6244	9.568	9.776
605	405	14.082		905	22.500		6117	12.525		6245	11.962	
	406	14.682	14.682	906	22.667	22.583	6118	12.400	12.462	6246	12.346	12.154
608	411	15.682		911	25.000		6119	14.000		6247	14.448	
	412	15.358	15.520	912	25.157	25.078	6120	13.568	13.784	6248	14.000	14.224
610	415	13.822		915	18.000		6121	12.000		6249	13.998	
	416	13.297	13.559	916	18.529	18.264	6122	12.466	12.233	6250	14.000	13.999
902	419	12.000		919	15.800		6123	8.000		6251	10.892	
	420	11.921	11.960	920	15.875	15.837	6124	7.578	7.789	6252	10.367	10.629
904	423	12.895		923	17.828		6127	11.100		6255	13.000	
	424	13.224	13.060	924	17.000	17.414	6128	10.862	10.981	6256	13.333	13.166

TABLE XX. THE AMMONIFICATION OF CASEIN.

I				II			III		
Plot No.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.	Lab. No.	Ammonia Mgs. N.	Av. Mgs. N.
601	1	68.54		49	61.12		137	55.38	
	2	68.23	68.38	50	60.52	60.82	138	55.98	55.67
602	3	71.87		51	63.39		139	59.31	
	4	71.26	71.56	52	63.55	63.47	140	59.31	59.31
606	5	78.68		53	69.59		141	64.55	
	6	78.83	78.74	54	69.59	69.59	142	63.55	64.05
607	7	76.65		55	66.44		143	63.09	
	8	74.14	74.89	56	66.26	66.35	144	63.24	63.15
609	9	lost		57	64.79		145	60.52	
	10	73.53	73.53	58	64.61	64.45	146	60.52	60.52
901	11	75.65		59	68.08		147	63.54	
	12	75.65	75.65	60	68.23	68.15	148	63.39	63.46
904	15	74.43		63	65.36		151	60.52	
	16	74.14	74.28	64	65.06	65.21	152	61.42	60.97

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STUDIES ON THE NUMBERS OF BACTERIA IN MILK SHOWING VARIOUS CHANGES

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Studies on the Numbers of Bacteria Present in Milk which has Undergone Various Changes

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The relationship between bacteria and the changes that occur in milk has been recognized since the middle of the last century. Certain of the milk abnormalities are almost characteristic of micro-organisms while others, such as abnormal flavors and odors, quite commonly result from causes other than bacterial. Recently the dairy section of the Iowa Agricultural Experiment Station in working with samples of milk from various parts of the state found that milk sometimes received low flavor scores when the number of contained bacteria was exceedingly small, while samples with high flavor scores sometimes contained large numbers of bacteria. This suggested an investigation of the number of organisms required to produce changes in the flavor and odor of milk.

The investigation of the odors and flavors produced in milk by bacteria, however, presents certain difficulties. The ordinary sterilization of milk so influences the flavor and odor that those which may be subsequently developed are largely overshadowed. Samples of aseptic milk sometimes have an undesirable flavor and odor that tends to mask other flavors that may be developed. Moreover, the accurate detection of changes in odor and taste presents difficulties because of the varying acuteness of the senses involved. Because of these difficulties much of the work herein reported deals with changes other than those in flavor and odor, such as the production of ropiness, the coagulation of milk and the reduction of litmus milk.

HISTORICAL.

Changes in milk have been studied by many investigators and a large number of types of organisms that can cause pronounced changes have been described. The type of change in milk depends on the type or types or organisms developing, which, in raw milk, is determined very largely by the temperature of holding.

Wide variations have been encountered in the changes in the numbers of bacteria in milk under apparently identical condi-

tions. Conn and Esten[1] have pointed out that "the number of bacteria which are present in fresh milk gives no indication as to the number that may be present in later hours. It frequently happens that milk which at the outset has small numbers will in later hours have numbers considerably larger than those in other samples which at the outset contained more bacteria, even tho the two samples of milk are kept under identical conditions."

The relationship between the numbers of bacteria present and the changes produced in milk has been considered with the lactic acid producers more than with any other group. The old idea regarding this relationship is stated by Conn[2] as follows: "Now while there is a general parallelism between the growth of these organisms and the production of lactic acid, the parallel is by no means a close one. For a considerable time after milk is inoculated with lactic bacteria, there is no increase in the amount of lactic acid, altho the bacteria are themselves multiplying very rapidly and becoming very numerous. Then there is a rapid increase in the development of lactic acid, accompanying a still further growth in the bacteria."

Rahn[3], after quoting the above statement by Conn as well as an analogous one by Knösel[4] which refers to a "short multiplication" preceding the sugar-destroying power of yeasts, contends that "there is no experiment on record to prove that in the first stage of development the multiplication takes place without fermentation. As soon as a determination of fermentation products is possible, it shows the fermentation per cell to be the faster the younger the culture." This author found that the fermenting capacity decreases with the age of the culture. Heinemann[5] found the amount of acid produced by the original strain of *Streptococcus lacticus* progressively decreased by animal passage; repeated transfers thru litmus milk did not change materially the fermentative ability. A decreased acid production after a culture has been transferred a number of times is a condition commonly met with in the laboratory.

Marshall[6] and his associates have pointed out that the other organisms present may influence the acid as well as the flavor and odor developed by *Bact. lactis acidi.* Buchanan and Hammer[7] have found that the inoculation, along with *Bact. lactis acidi,* of an organism isolated by them decreased the acid formed.

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1. The Comparative Growth of Different Species of Bacteria in Normal Milk. 14th Annual Rpt. Storrs Agr. Expt. Sta. 76. 1901.
2. Bacteria in Milk and its Products. 74. 1903.
3. The Fermenting Capacity of the Average Single Cell of Bacterium lactis acidi. 24th Annual Rpt. Mich. Agr. Expt. Sta. 443-480. 1911.
4. Centbl. f. Bakt. Abt. 2, 8: 272.
5. The Variability of two Strains of Streptococcus lacticus. Jour of Inf. Dis. 16: 221-239. Mr. 1915.
6. Various Bulletins of the Mich. Agr. Expt. Sta.
7. Slimy and Ropy Milk. Res. Bull. Ia. Agr. Expt. Sta. 22.

Heinemann[8] found that "The amount of acid formed in the souring process of milk or cream is not dependent solely on a definite number of bacteria of the *Streptococcus lacticus* group. Temperature and the presence of other bacteria may influence the result." He also found that coagulation is not dependent solely on definite numbers of bacteria or a definite amount of acid and suggests that acid forming and coagulative enzymes excreted by bacteria or liberated after disintegration of the cells, play an important role in bringing about coagulation. In sterilized milk, this author found that coagulation was complete with a smaller amount of acid than inoculated raw milk, but that more time was required for completion of the process.

Heinemann[9] also states that "we did some work in trying to determine the number of lactic acid streptococci that are necessary in order to change milk and cream in such a way that you could tell by taste whether it was sour or not. In a general way, the temperature influences this. The higher the temperature the fewer the bacteria (that) are necessary to sour milk."

A number of investigators have found wide variations between the acidities and bacterial contents of samples of milk showing acidities above the normal and Hammer[10] found these variations in samples of sour cream delivered to one of the Iowa creameries.

Heinemann[11] found in sterilized milk inoculated with *Streptococcus lacticus* that at 37° C. the milk was coagulated after about 36 hours and contained 540,000,000 per c. c. while at room temperature coagulation took place after 4 days with 350,000,000 per c. c.

Rahn[12] has observed an acidity increase with *Bact. lactis acidi* where the number per c. c. was as low as 4,800,000; the rise in acidity, however, was only .005%.

It is evident that wide variations apparently exist in the action of a given number of a certain type of organism; evidently the variation is much greater in impure culture than in pure culture, altho it undoubtedly exists in the latter case. Certain factors, such as temperature and character of the milk, undoubtedly play a part in causing this variation.

Marshall and Farrand[13] write as follows regarding another possible cause: 'We are disposed from our studies to attribute these irregularities to associative action . . . . ."

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8. Relation of the Number of Streptococcus lacticus to the Amount of Acid Formed in Milk and Cream. Jour. of Inf. Dis. 16: 285-291. Mr. 1915.
9. Rpt of the Hearing on Ice Cream Before Dr. C. A. Alsberg. 120. Je. 1914. (Published by the Nat'l Assn. of Ice Cream Mfrs.)
10. Pasteurization of Cream for Buttermaking. Part II Bacteriological Studies. Bull. Ia. Agr. Expt. Sta. 156.
11. l. c. ref. 8.
12. l. c.
13. Bacterial Associations in the Souring of Milk Centbl. f. Bakt. 2. 21-58 1908.

GENERAL METHODS.

In general, the method of procedure was to inoculate milk, either sterile or aseptic, with a pure culture of some organism producing a change in milk and, when this change was detected, to determine the number of bacteria present by the plate method.

The sterile milk used, both litmus and plain, was sterilized in the autoclave; the use of intermittent sterilization, even with temperature as low as 0° C., failed to show any advantage over autoclaving, from the standpoint of the production of a heated flavor and odor. The aseptic milk used was secured at the Iowa State College dairy farm. Previous work on the bacterial content of the milk of some of the animals in the herd made possible the selection of animals whose milk ordinarily contained but few bacteria. When plates were poured from this milk after inoculating various organisms and allowing time for development, the colonies developing were ordinarily practically all of the type inoculated.

Most of the data were secured by using tubes of milk altho a few tests were run with Petri dishes. The amount of milk used varied, altho some tests were run with 10 c. c. quantities. The numbers of organisms used for inoculation were not determined; where comparative inoculations were desired equal volumnes of a suspension were employed.

The agar of the American Public Health association for bacterial milk analysis was used in all of the plates, which were all incubated at 37° C. for 48 hours with the exception of those poured with *Bact. lactis viscosum*, which were inoculated at room temperature for 48 hours. After a change had taken place in inoculated milk, the whole volume was thoroly agitated before the sample for plating was taken. The inoculated milk was commonly held at room temperature, altho a few tests were run at 37° C. Room temperature with its customary variation seemed to present more practical conditions than any constant temperature, altho a constant temperature might have given more uniform results.

The classification of the changes produced in milk by the various organisms presented considerable difficulty; the changes recognized for each organism are given in the methods used with that type. The methods of detecting changes, such as slight ropiness (touching the milk with a platinum wire) or slight coagulation (by tipping the tube) are quite unsatisfactory. Moreover, it does not seem desirable to apply these methods too frequently because of the effect of the agitation involved on the ropiness and on the tendency of the curd to cling to the bottom of the test tube. Accordingly, in the work on the

changes in the consistency of milk, examinations were only made at from 2 to 4 hour intervals; in the work on odor and flavor the intervals were ordinarily shorter. On account of the difficulties involved, any classification of the changes that is adopted can be only approximately adhered to. Because of the wide variations in the results, the averages for the various sets of data are given in general rather than in exact figures.

The cultures of *Bact. lactis acidi* and the sweet curdlers used were isolated for the work herein reported while the cultures of the other organisms employed were cultures that had been held for varying lengths of time. In many of the tables presented, the determinations were all, or nearly all, made on different days so that a considerable period of time and various conditions are represented. The results obtained on *Bact. lactis acidi* and the sweet curdlers shortly after isolation are essentially the same as those obtained later, so it seems probable that the holding of the organisms in pure culture for considerable periods did not exert an appreciable influence on the results; nevertheless the history of the cultures must be taken into consideration in drawing conclusions from the data given. The problem herein considered is being investigated at the present time by studying the numbers of organisms in milk that has undergone various changes without artificial inoculation. With certain changes, the depth of the milk layer involved will undoubtedly be of some influence.

RESULTS OBTAINED

Bact. lactis aerogenes

The culture of *Bact. lactis aerogenes* that was used was isolated from a sample of ropy milk. It produced an extremely ropy condition in milk and retained this ability after being carried in sterile milk for a number of years. The organism was studied simply from the standpoint of its production of ropiness.

SPECIAL METHODS

All the work with *Bact. lactis aerogenes* was done with sterile milk. Generally tubes containing from 8 to 15 c. c. of milk were used, but in a few instances Petri dishes (with from 8 to 15 c. c. of milk) or 150 c. c. Erlenmeyer flasks (with 100 c. c. milk) were employed; unless otherwise stated, tubes were used. Room temperature was always used as a holding temperature for the inoculated milk. Ropiness in the inoculated milk was detected by touching a flamed and cooled platinum needle to the surface, the container being so held that the surface of the milk made an acute angle with the needle, and then carefully

withdrawing it. The fermented milk was classified as follows:

(A) Slightly Ropy: Milk showing only a slight tendency to follow the platinum needle when it was withdrawn from the surface.

(B) Medium Ropy: Milk which could be drawn out into threads extending 2 or 3 inches from the surface.

(C) Very Ropy: Milk which could be drawn out into threads, extending beyond the mouth of the tube, but which could not be included in the following class.

(D) Extremely Ropy: Milk which could be drawn out into threads several feet long.

RESULTS OBTAINED

The counts obtained on milk which was classed as slightly ropy are shown in tables I and II, the data having been divided because of the very wide variations existing. Table I gives the results of seven determinations which vary from 11,000,000 to 49,000,000 per c. c. and average 32,200,000; the ages of the cultures range from 8 to 36 hours. Table II presents the results of 37 determinations which vary from 79,000,000 to 156,000,000 and average 115,700,000 per c. c., the ages of the cultures ranging from 9 to 36 hours. Table III presents data obtained on samples of milk classed as medium ropy; the 36 counts range from 160,000,000 to 285,000,000 per c. c. and average 223,000,000 while the ages of the cultures vary from 9 to 72 hours. The counts obtained on the samples of milk classed as very ropy are presented in table IV; the 37 counts vary from 290,000,000 to 495,000,000 per c. c. and average 373,000,000 while the ages of the cultures range from 8 to 72 hours. Table V presents the data secured on milk (in tubes) that was classed as extremely ropy. The nine counts range from 500,000,000 to 830,000,000 per c. c. and average 650,000,000, while the ages of the cultures vary from 12 to 24 hours. The data obtained on extremely ropy milk fermented in thin layers (in Petri dishes) is presented in table VI; the average for the 5 counts is 3,800,000,000 per c. c. and the individual counts vary from 2,870,000,000 to 4,960,000,000, while the ages of the cultures range from 8 to 12 hours. Table VII presents data relative to the numbers of bacteria present in inoculated milk at 2 hour periods, the determination having been made on milk held in flasks. Organisms were present up to 86,000,000 per c. c. without ropiness being detected. There was considerable variation in the numbers of organisms present at the time the milk was slightly ropy and some of the figures are somewhat higher than those reported in tables I to VII. The number of organisms which produced a slightly ropy condition in trial 5 is smaller than the

TABLE I—Bact. lactis aerogenes. Number of Organisms in Slightly Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 11, 1914..	24	22,000,000
Jan. 10, 1915..	12	11,000,000
Jan. 30, 1915..	10	49,000,000
Feb. 1, 1915..	12	38,000,000
Feb. 15, 1915..	36	49,000,000
March 18, 1915	9	42,000,000
March 30, 1915	8	14,400,000
	Average	32,200,000

TABLE II—Bact. lactis aerogenes. Number of Organisms in Slightly Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 2, 1914..	24	156,000,000
Nov. 2, 1914..	24	80,000,000
Nov. 13, 1914..	24	114,000,000
Nov. 13, 1914..	24	130,000,000
Nov. 16, 1914..	15	81,000,000
Nov. 19, 1914..	24	90,000,000
Nov. 21, 1914..	24	105,000,000
Nov. 21, 1914..	24	105,000,000
Nov. 22, 1914..	24	155,000,000
Nov. 23, 1914..	24	111,000,000
Dec. 12, 1914..	24	80,000,000
Dec. 13, 1914..	24	84,000,000
Dec. 14, 1914..	24	129,000,000
Jan. 18, 1915..	12	150,000,000
Jan. 19, 1915..	12	151,000,000
Jan. 21, 1915..	12	109,000,000
Jan. 22, 1915..	12	95,000,000
Jan. 22, 1915..	12	156,000,000
Jan. 22, 1915..	12	142,000,000
Jan. 23, 1915..	12	79,000,000
Jan. 26, 1915..	12	137,000,000
Jan. 28, 1915..	12	136,000,000
Jan. 31, 1915..	24	132,500,000
Feb. 3, 1915..	12	151,000,000
Feb. 5, 1915..	12	110,000,000
Feb. 6, 1915..	12	87,000,000
Feb. 15, 1915..	36	113,000,000
Feb. 17, 1915..	12	84,000,000
Feb. 21, 1915..	12	101,000,000
Feb. 26, 1915..	12	134,000,000
March 2, 1915	12	93,000,000
March 3, 1915	12	125,000,000
March 8, 1915	12	117,000,000
March 20, 1915	9	100,000,000
March 24, 1915	9	99,000,000
March 26, 1915	9	150,000,000
March 26, 1915	9	105,000,000
	Average	115,700,000

TABLE III—Bact. lactis aerogenes. Number of Organism in Medium Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 26, 1914..	24	280,000,000
Oct. 29, 1914..	24	208,000,000
Oct. 30, 1914..	24	280,000,000
Oct. 31, 1914..	24	180,000,000
Nov. 7, 1914..	18	230,000,000
Nov. 5, 1914..	18	186,000,000
Nov. 8, 1914..	72	285,000,000
Nov. 25, 1914..	24	166,000,000
Nov. 27, 1914..	24	226,000,000
Nov. 28, 1914..	48	165,000,000
Nov. 29, 1914..	24	150,000,000

TABLE III—Continued.

Date	Age of Culture in Hours	No. Bact. per c. c.
Dec. 10, 1914..	24	257,000,000
Dec. 11, 1914..	24	204,000,000
Dec. 7, 1914..	18	188,000,000
Dec. 18, 1914..	18	245,000,000
Dec. 21, 1914..	18	175,000,000
Jan. 1, 1915..	18	270,000,000
Jan. 11, 1915..	24	250,000,000
Jan. 14, 1915..	12	160,000,000
Jan. 15, 1915..	12	238,000,000
Jan. 15, 1915..	12	233,000,000
Jan. 15, 1915..	12	260,000,000
Jan. 16, 1915..	12	260,000,000
Jan. 16, 1915..	12	188,000,000
Jan. 17, 1915..	12	190,000,000
Jan. 18, 1915..	12	225,000,000
Feb. 2, 1915..	12	228,000,000
Feb. 9, 1915..	12	181,000,000
Feb. 11, 1915..	12	269,000,000
Feb. 23, 1915..	12	228,000,000
Feb. 24, 1915..	12	262,000,000
Feb. 27, 1915..	12	230,000,000
March 4, 1915	12	272,000,000
March 5, 1915	12	272,000,000
March 15, 1915	12	210,000,000
March 22, 1915	9	187,000,000
	Average	225,000,000

TABLE IV—Bact. lacti's aerogenes. Number of Organisms in Very Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 17, 1914..	18	440,000,000
Oct. 18, 1914..	16	320,000,000
Oct. 19, 1914..	18	400,000,000
Oct. 22, 1914..	18	300,000,000
Oct. 23, 1914..	24	310,000,000
Oct. 25, 1914..	24	370,000,000
Oct. 26, 1914..	24	370,000,000
Oct. 28, 1914..	24	300,000,000
Nov. 1, 1914..	24	320,000,000
Nov. 4, 1914..	24	370,000,000
Nov. 6, 1914..	18	360,000,000
Nov. 8, 1914..	72	425,000,000
Nov. 9, 1914..	24	315,000,000
Nov. 12, 1914..	24	324,500,000
Nov. 14, 1914..	24	367,000,000
Nov. 20, 1914..	24	330,000,000
Nov. 26, 1914..	24	339,000,000
Nov. 29, 1914..	18	450,000,000
Dec. 2, 1914..	24	405,000,000
Dec. 3, 1914..	24	415,000,000
Dec. 5, 1914..	24	465,000,000
Dec. 6, 1914..	24	370,000,000
Dec. 7, 1914..	24	430,000,000
Jan. 10, 1915..	24	450,000,000
Jan. 13, 1915..	18	495,000,000
Jan. 17, 1915..	12	390,000,000
Jan. 18, 1915..	12	360,000,000
Jan. 20, 1915..	12	360,000,000
Feb. 5, 1915..	24	420,000,000
Feb. 12, 1915..	36	360,000,000
Feb. 13, 1915..	18	365,000,000
Feb. 21, 1915..	12	400,000,000
Feb. 22, 1915..	12	338,000,000
Feb. 25, 1915..	12	315,000,000
March 11, 1915	8	290,000,000
March 27, 1915	24	428,000,000
March 30, 1915	8	320,000,000
	Average	373,000,000

TABLE V—Bact. lactis aerogenes. Number of Organisms in Extremely Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 30, 1914..	24	565,000,000
Dec. 9, 1914..	24	710,000,000
Dec. 23, 1914..	24	830,000,000
Dec. 31, 1914..	24	695,000,000
Jan. 2, 1915..	24	580,000,000
Jan. 7, 1915..	24	690,000,000
Jan. 8, 1915..	24	750,000,000
Jan. 26, 1915..	15	530,000,000
March 6, 1915	12	500,000,000
	Average	650,000,000

TABLE VI—Bact. lactis aerogenes. Number of Organisms in Extremely Ropy Milk in Petri Dishes.

Date	Age of Culture in Hours	No. Bact. per c. c.
March 8, 1915	12	3,880,000,000
March 10, 1915	12	3,290,000,000
March 11, 1915	12	4,000,000,000
March 16, 1915	12	2,870,000,000
March 30, 1915	8	4,960,000,000
	Average	3,800,000,000

TABLE VII—BACT. LACTIS AEROGENES.

Age of Culture in hours	TRIAL 1.		TRIAL 2.		TRIAL 3.	
	Bacteria per c. c.	Cond. of Milk	Bacteria per c. c.	Cond. of Milk	Bacteria per c. c.	Cond of Milk
2	4,400,000	no change	12,350,000	no change	14,600,000	no change
4	25,300,000	no change	42,400,000	no change	22,500,000	no change
6	80,000,000	no change	86,000,000	no change	44,000,000	no change
8	186,000,000	slightly ropy	202,500,000	slightly ropy	248,000,000	slightly ropy
10	530,000,000	extremely ropy				

TRIAL 4.		TRIAL 5.	
Bacteria per c. c.	Cond. of Milk	Bacteria per c. c.	Cond. of Milk
8,900,000	no change	7,500,000	no change
60,000,000	no change	12,900,000	no change
348,000,000	med. ropy	25,000,000	no change
500,000,000	very ropy	70,000,000	slightly ropy
		125,000,000	med. ropy

numbers present in trials 1 and 2 when there was no ropiness present.

The results obtained on *Bact. lactis aerogenes* indicate that under the conditions employed, a very large number of organisms must be present in milk before a ropy condition results. The smallest number of organisms encountered in milk that was at all ropy was 11,000,000 per c. c. and this was an exceptional case. In many instances over 100,000,000 organisms per c. c. were present in the milk when ropiness was first detected. On the other hand numbers up to 86,000,000 per c. c. were found present in milk without ropiness having developed and since the data on this point are not extensive, the figures probably by no means represent extremes. For the development of an extremely ropy condition in milk exceedingly large numbers of organisms, ordinarily in the hundreds of millions, must be present. Wide

variations regarding the numbers of organisms present in milk classed as showing the same stage of ropiness were constantly encountered; while this is undoubtedly due in part to the difficulty and uncertainty of the method used for the classification, it may be in part due to the effect of different temperatures on the organism. High temperatures commonly limit or prevent the development of ropiness by certain bacteria altho growth goes on rapidly; the more rapid development of acid at higher temperatures tends to reduce the ropiness, but there is likely some direct effect on the organisms also.

BACT. LACTIS VISCOSUM

The culture of *Bact. lactis viscosum* employed was isloted from an outbreak of ropy milk in one of the smaller towns of the state. Altho the culture had been carried in litmus milk for several months at the time it was selected, it produced a very ropy condition in milk altho not so ropy as the culture of *Bact. lactis aerogenes* used. *Bact. lactis viscosum* was studied only from the standpoint of its production of ropiness in milk.

SPECIAL METHODS

Tubes containing from 8 to 15 c. c. of sterilized milk were used for all of the work with this organism. The cultures (and plates also as already stated) were incubated at room temperature; ropiness was tested for in the same manner as with *Bact. lactis aerogenes*. The fermented milk was classified as follows:

(A) Slightly Ropy: Milk which showed only a slight tendency to follow a platinum needle withdrawn from the surface of the milk.

(B) Very Ropy: Milk which could be drawn out into threads several inches long.

RESULTS OBTAINED

The counts obtained on the milk classed as slightly ropy are presented in table VIII; the 19 determinations range from 15,500,000 to 44,000,000 per c. c. and average 26,100,000, while the ages of the cultures range from 9 to 24 hours. Table IX gives the data obtained on very ropy milk; the four counts, which were all made on the same day, range from 450,000,000 to 786,000,000 and average 556,000,000 per c. c., while the age of all of the culture was 24 hours.

Table X gives the data obtained when cultures were plated at two hour periods from the time of inoculation until ropiness was detected. The numbers of organisms present at the time a slight ropiness was detected were in general the same as those

given in table VIII, altho the range was somewhat greater than the range encountered there. The lowest number of organisms found in slightly ropy milk was 12,300,000 per c. c. while numbers larger than that (up to 19,500,000 per c. c.) were found in milk that showed no evidence of ropiness.

The results obtained with *Bact. lactis viscosum* indicate that under the conditions employed, a large number of organisms must be present in milk before a ropy condition is produced. Slightly ropy milk showed a smaller variation in the numbers of organisms present with *Bact. lactis viscosum* than with *Bact. lactis aerogenes*. The smallest number in ropy milk was 12,300,00 per c. c. when *Bact. lactis viscosum* was used for inoculation; this value agrees very closely with the corresponding value of 11,000,000 per c. c. obtained with *Bact. lactis aerognes*. With

TABLE VIII.—Bact. lactis viscosum. Number of Organisms in Slightly Ropy Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 30, 1914.	24	21,000,000
Dec. 10, 1914.	24	29,500,000
Dec. 2, 1914.	24	44,000,000
March 23, 1915.	9	21,500,000
March 24, 1915.	9	38,000,000
March 26, 1915.	9	39,000,000
March 27, 1915.	9	21,000,000
March 27, 1915.	9	20,000,000
March 31, 1915.	24	37,600,000
April 16, 1915.	12	18,750,000
April 16, 1915.	12	15,500,000
April 16, 1915.	12	20,200,000
April 16, 1915.	12	28,300,000
April 21, 1915.	9	18,150,000

TABLE VIII -Continued.

Date	Age of Culture in Hours	No. Bact. per c. c.
April 21, 1915.	9	25,000,000
April 21, 1915.	9	23,750,000
April 21, 1915.	9	41,200,000
April 21, 1915.	12	17,500,000
April 21, 1915.	9	15,800,000
	Average	26,100,000

TABLE IX—Bact. lactis viscosum. Number of Organisms in Very Ropy Milk.

June 25, 1915.	24	520,000,000
June 25, 1915.	24	470,000,000
June 25, 1915.	24	450,000,000
June 25, 1915.	24	786,000,000
	Average	556,000,000

TABLE X—BACT. LACTIS VISCOSUM.

Age of Culture in hours	TRIAL 1. Bacteria per c. c.	TRIAL 1. Cond. of Milk	TRIAL 2. Bacteria per c. c.	TRIAL 2. Cond. of Milk	TRIAL 3. Bacteria per c. c.	TRIAL 3. Cond. of Milk
2	1,860,000	no change	2,200,000	no change	2,780,000	no change
4	6,400,000	no change	5,480,000	no change	7,500,000	no change
6	12,300,000	slightly ropy			10,950,000	no change
8	14,000,000	slightly ropy	24,000,000	slightly ropy	30,500,000	slightly ropy

TRIAL 4. Bacteria per c. c.	TRIAL 4. Cond. of Milk	TRIAL 5. Bacteria per c. c.	TRIAL 5. Cond. of Milk
8,800,000	no change	5,840,000	no change
		16,800,000	no change
15,600,000	no change	19,500,000	no change
62,500,000	slightly ropy	21,050,000	slightly ropy

Bact. lactis viscosum very ropy milk contained large numbers of organisms in the few cases studied, 450,000,000 per c. c. representing the minimum and 786,000,000, the maximum.

BITTER ORGANISM

The bitter organism employed was isolated in the student dairy bacteriology laboratory. It causes a coagulation of milk, begining at the bottom and gradually extending upward, a bitter taste, an increase in the acidity and eventually a digestion of the curd. Acidity determinations made at the time of coagulation indicate that acid is not responsible for the precipitation of the casein. The organism was encountered only once but an apparently identical organism has been supplied to this laboratory by Prof. E. G. Hastings. A considerable portion of the work on this organism dealt with the numbers of organisms present at the various stages of coagulation; the number of organisms present in aseptic milk at the time bitterness is first evident was, however, determined in a number of trials.

SPECIAL METHODS

The work on coagulation was done with tubes of sterile milk (8 to 15 c. c. amounts). The aseptic milk used was drawn directly into large test tubes in amounts varying from 30 to 50 c. c. All the inoculated milk was held at room temperature. The coagulation of the milk was observed by tipping the tubes and allowing the uncoagulated milk to flow against the stopper; the stages of coagulation were classed as follows:

(A) Very Slight Coagulation: Milk in which coagulation had just definitely taken place, a very small amount of curd being present.

(B) Slight Coagulation: Milk showing a stage of coagulation somewhere between very slight coagulation and the following stage.

(C) Base Coagulation: Milk that had curdled up as far as sides of the tube.

(D) Nearly Complete Coagulation: Milk which was curdled for about one inch up from the bottom of the tube.

(E) Freshly Coagulated Cultures: Milk in which coagulation had been completed with a few hours.

(F) Old Coagulated Cultures: Milk held from 24 to 48 hours after complete coagulation.

RESULTS OBTAINED

Table XI gives the data obtained on the milk showing very slight coagulation. The average of the 18 determinations is

TABLE XI.—Bitter Organisms.
Numbers of Organisms in Very Slightly Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Dec. 6, 1914..	24	68,000,000
Jan. 1, 1915..	18	46,000,000
Jan. 31, 1915..	12	30,000,000
Feb. 1, 1915..	12	51,000,000
Feb. 2, 1915..	12	43,000,000
Feb. 3, 1915..	12	41,000,000
Feb. 11, 1915..	36	59,000,000
Feb. 23, 1915..	12	47,000,000
March 4, 1915	12	47,000,000
March 8, 1915	12	22,000,000
March 18, 1915	9	56,000,000
March 18, 1915	9	39,000,000
March 19, 1915	9	41,000,000
March 20, 1915	9	68,000,000
March 22, 1915	9	29,000,000
March 26, 1915	9	34,000,000
March 26, 1915	9	50,000,000
March 28, 1915	12	22,000,000
	Average	44,000,000

TABLE XII—Bitter Organism.
Number of Organisms in Slightly Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 29, 1914..	24	110,000,000
Oct. 30, 1914..	24	90,500,000
Nov. 20, 1914..	24	152,000,000
Dec. 11, 1914..	24	131,000,000
Dec. 12, 1914..	24	138,000,000
Dec. 14, 1914..	24	135,000,000
Dec. 17, 1914..	18	84,000,000
Dec. 19, 1914..	18	152,000,000
Jan. 8, 1915..	18	116,000,000
Jan. 11, 1915..	24	113,000,000
Jan. 17, 1915..	12	141,000,000
Jan. 18, 1915..	12	87,000,000
Jan. 19, 1915..	12	156,000,000
Jan. 21, 1915..	12	118,000,000
Jan. 22, 1915..	12	119,000,000
Jan. 25, 1915..	12	86,000,000
Feb. 5, 1915..	10	107,000,000
Feb. 5, 1915..	12	125,000,000
Feb. 15, 1915..	36	117,000,000
Feb. 15, 1915..	12	113,000,000
Feb. 21, 1915..	12	106,000,000
Feb. 26, 1915..	12	157,000,000
Feb. 26, 1915..	12	167,000,000
Feb. 26, 1915..	12	89,000,000
March 3, 1915	12	100,000,000
March 6, 1915	12	85,000,000
March 31, 1915	24	114,000,000
	Average	119,000,000

TABLE XIII—Bitter Organism.
Number of Organisms in Base Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 31, 1914..	24	267,000,000
Nov. 2, 1914..	24	260,000,000
Nov. 5, 1914..	24	350,000,000
Nov. 6, 1914..	18	181,000,000
Nov. 7, 1914..	18	265,000,000
Nov. 12, 1914..	48	280,000,000
Nov. 25, 1914..	24	320,000,000
Nov. 30, 1914..	24	350,000,000
Dec. 2, 1914..	24	295,000,000

TABLE XIII—Continued

Date	Age of Culture in Hours	Strain	No. Bact. per c. c.
Dec. 3, 1914..	24		250,000,000
Dec. 3, 1914..	24		373,000,000
Dec. 7, 1914..	24		200,000,000
Dec. 13, 1914..	24		285,000,000
Dec. 18, 1914..	18		250,000,000
Dec. 21, 1914..	18		340,000,000
Dec. 23, 1914..	24		375,000,000
Jan. 9, 1915..	18		270,000,000
Jan. 13, 1915..	18		370,000,000
Jan. 26, 1915..	18		280,000,000
Jan. 28, 1915..	12		300,000,000
Feb. 10, 1915..	24		310,000,000
Feb. 13, 1915..	18		210,000,000
Feb. 17, 1915..	12		310,000,000
Feb. 18, 1915..	12		283,000,000
Feb. 22, 1915..	12		280,000,000
March 10, 1915	12		270,000,000
	Average		289,000,000

TABLE XIV—Bitter Organism.
Number of Organisms in Nearly Completely Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 17, 1914..	24	445,000,000
Oct. 18, 1914..	24	550,000,000
Oct. 19, 1914..	18	650,000,000
Oct. 28, 1914..	24	470,000,000
Nov. 9, 1914..	24	510,000,000
Nov. 26, 1914..	24	690,000,000
Nov. 29, 1914..	24	600,000,000
Dec. 9, 1914..	24	630,000,000
Dec. 10, 1914..	24	600,000,000
Jan. 2, 1915..	24	415,000,000
Feb. 9, 1915..	24	610,000,000
March 5, 1915	12	450,000,000
March 8, 1915	12	479,000,000
	Average	545,000,000

TABLE XV.—Bitter Organism.
Number of Organisms in Freshly Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 23, 1914..	24	1,250,000,000
Oct. 25, 1914..	24	1,500,000,000
Oct. 26, 1914..	24	950,000,000
Nov. 1, 1914..	24	720,000,000
Nov. 4, 1914..	24	710,000,000
Nov. 13, 1914..	48	1,115,000,000
Dec. 31, 1914..	24	790,000,000
Jan. 7, 1915..	24	1,110,000,000
Feb. 12, 1915..	36	890,000,000
March 27, 1915	24	1,000,000,000
	Average	1,000,000,000

TABLE XVI—Bitter Organism.
Number of Organisms in Old Coagulated Milk.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 6, 1914..	72	2,515,000,000
Nov. 8, 1914..	48	2,060,000,000
Nov. 28, 1914..	48	2,365,000,000
Dec. 2, 1914..	48	2,290,000,000
Jan. 7, 1915..	48	2,400,000,000
	Average	**2,300,000,000**

44,000,000 per c. c., the individual counts varying from 22,000,000 to 68,000,000; the ages of the cultures range from 9 to 36 hours. The results obtained on milk showing slight coagulation are shown in table XII where the 27 counts vary from 84,000,000 to 167,000,000 per c. c. and average 119,000,000. The ages of the cultures vary from 10 to 36 hours, the majority of them being 12 hours.

The counts for the stage recognized as base coagulation are given in table XIII. The average of the 26 counts is 289,000,000 per c. c., while the individual counts range from 181,000,000 to 375,000,000. The ages of the cultures vary from 12 to 48 hours. The milk classed as nearly completely coagulated is represented in table XIV where the average for the 13 determinations is 545,000,000 per c. c. while the individual counts vary from 415,000,000 to 690,000,000; the ages of the cultures range from 12 to 24 hours. Table XVI gives the data obtained on the milk that was freshly coagulated. The average for the 10 determinations is 1,000,000,000 per c. c. while the individual counts vary from 710,000,000 to 1,500,000,000. The ages of the cultures represented vary from 24 to 48 hours. The data dealing with the old coagulated milk is shown in table XVI. The average for the five determinations is 2,300,000,000 per c. c. while the individual counts vary from 2,060,000,000 to 2,515,000,000. All of the cultures except one were 48 hours old and that was 72 hours old. Table XVII gives the data obtained on inoculated cultures at two hour intervals. In general the counts agree fairly well with those obtained in the preceding tables. The lowest count recorded in table XVII as causing very slight coagulation is 31,000,000 per c. c. (22,000,000 per c. c. is recorded in table X); in another trial 39,000,000 organisms per c. c. were found without any change being evident in the milk.

The data obtained relative to the development of a bitter-

TABLE XVII—BITTER ORGANISM

Number of Organisms at two-hour Periods.

Age of Culture in Hours	Trial 1		Trial 2		Trial 3	
	Bacteria per c. c.	Cond. of Milk	Bacteria per c. c.	Cond. of Milk	Bacteria per c. c.	Cond. of Milk
2	313.000	no change	850.000	no change	350.000	no change
4	3,500.000	no change	2,100.000	no change	7,800.000	no change
6	39,000.000	no change	13,000.000	no change	10,200,000	no change
8	51,000.000	very slight coag.	31,000.000	very slight coag.	44,000.000	very slight coag.
10	200,000.000	slight coag.	87,000.000	slight coag.		
12	241,000.000	base coag.				

TABLE XVIII—BITTER ORGANISM ASEPTIC MILK

Number of Organisms in Slightly Bitter Milk.

Date	Cow Number	Age of Culture						Bacteria per c. c.
June 23, 1915.	69	15	Slightly	Bitter	Very	Slight	Coag.	63,000,000
June 23, 1915.	87	15	Slightly	Bitter	Very	Slight	Coag.	60,000,000
June 23, 1915.	207	18	Slightly	Bitter	Very	Slight	Coag.	42,000,000
June 23, 1915.	143	15	Slightly	Bitter	Very	Slight	Coag.	59,000,000
June 24, 1915.	207	18	Slightly	Bitter	no	Coagulation		19,000,000
June 24, 1915.	143	18	Slightly	Bitter	no	Coagulation		10,000,000
June 24, 1915.	213	18	Slightly	Bitter	no	Coagulation		66,000,000
June 24, 1915.	69	18	Slightly	Bitter	no	Coagulation		23,000,000
June 24, 1915.	87	15	Slightly	Bitter	no	Coagulation		11,000,000
June 24, 1915.	162	15	Slightly	Bitter	no	Coagulation		35,000,000
							Average	38,900,000

ness in aseptic milk is shown in table XVIII; the 10 counts vary from 10,000,000 to 66,000,000 and average 38,900,000 per c. c. The samples studied on the first day all showed a very slight coagulation while those studied on the second day did not; most of the counts obtained on the second day were lower than those obtained on the first day, but one of those of the second day was a little higher than any of those of the first day.

The results obtained with the bitter organism indicate that large numbers of organisms must be present in milk before coagulation or bitterness are produced. The smallest number of organisms found in a culture showing very slight coagulation was 22,000,000 per c. c. while 10,000,000 per c. c. was the smallest number found in aseptic milk in which bitterness could be detected. In general, with an increase in the extent of coagulation, there is an increase in the number of organisms present, altho in some cultures having no coagulation numbers of organganisms are found which are greater than the number found in other cultures which do show coagulation. Wide variations are encultures which do show coagulation. Wide variations are encountered in the numbers of organisms present in cultures showing the same stage of coagulation. In freshly coagulated cultures, exceedingly large numbers of organisms are found—commonly over 1,000,000,000 per c. c. After coagulation the development of the organisms still goes on, as in the old coagulated cultures over 2,000,000,000 per c. c. are ordinarily found.

Sweet Curdlers

The three sweet curdling organisms used, which were classed as *B. subtilis* after a rather brief study of their characteristics, were isolated from three samples of milk. The three strains (A, B, and C), all of which sweet curdled milk rapidly, were studied simply from the stand point of the numbers of organisms present in milk in different stages of coagulation.

SPECIAL METHODS

The work was all done with tubes of sterile milk containing from 8 to 15 c. c. The inoculated tubes were held at 37° C. in the majority of the cases, altho a few trials were made at room temperature. The following stages in the coagulation of the milk were recognized:

(A) Slight Coagulation: Milk which was just thickening.

(B) Medium Coagulation: Milk which was well curdled but not yet firmly set.

(C) Firm Coagulation: Milk which was quite firmly set.

RESULTS OBTAINED

Table XIX shows the data obtained on the milk classed as showing slight coagulation. Four of the tests were run at room temperature and the remaining 17 at 37° C., but the uniformity of the counts apparently justifies the combining of the data. The average for the 21 counts is 3,100,000 per c. c. while the individual counts vary from 1,250,000 to 4,900,000. The ages of the room temperature cultures vary from 24 to 48 hours and those of the 37° C. cultures from 9 to 24 hours. The numbers of organisms present in milk classed as showing medium coagulation are presented in table XX. Seven of the counts were on tubes held at room temperature, while the remaining 15 were on tubes held at 37° C.; here again the uniformity of the counts makes it seem advisable to combine the data. The 22 counts average 9,300,000 per c. c. and vary from 6,200,000 to 14,600,00. The ages of the room temperature cultures range from 12 to 48 hours and of the 37° C. cultures from 9 to 24 hours. Table XXI presents the

TABLE XIX—SWEET CURDLERS

Number of Organisms in Slightly Coagulated Milk.

Date	Age of Culture in Hours	Strain	Temperature Held	Bact. per c. c.
December 31, 1914	24	A	37°	4,900,000
December 31, 1914	24	B	37°	2,600,000
January 7, 1915	24	A	37°	3,600,000
January 8, 1915	18	C	37°	1,400,000
January 9, 1915	18	A	37°	1,400,000
January 16, 1915	24	C	37°	3,800,000
January 16, 1915	24	C	37°	3,800,000
January 19, 1915	12	A	37°	1,900,000
January 19, 1915	12	B	37°	4,000,000
January 21, 1915	12	C	37°	4,600,000
January 23, 1915	12	C	37°	3,850,000
January 25, 1914	24	A	37°	1,700,000
January 28, 1915	12	A	37°	3,700,000
January 31, 1915	24	A	37°	4,800,000
February 1, 1915	48	A	Room	3,000,000
February 9, 1915	48	C	Room	1,250,000
February 10, 1915	24	A	Room	3,000,000
February 11, 1915	12	C	37°	4,200,000
February 20, 1915	12	C	37°	3,700,000
March 18, 1915	9	A	37°	1,400,000
April 19, 1915	24	A	Room	3,190,000
			Average	3,100,000

TABLE XX—SWEET CURDLERS—NUMBER OF ORGANISMS IN MEDIUM COAGULATED MILK.

Date	Age of Culture in Hours	Strain	Temperature Held	No. Bact. per c. c.
January 1, 1915	18	A	37°	7,000,000
January 2, 1915	18	A	37°	13,000,000
January 8, 1915	18	A	37°	8,750,000
January 12, 1915	18	B	37°	8,850,000
January 15, 1915	48	A	Room	9,700,000
January 15, 1915	48	B	Room	10,700,000
January 16, 1915	24	A	37°	6,500,000
January 22, 1915	24	A	37°	13,200,000
January 26, 1915	48	C	Room	6,250,000
February 15, 1915	24	C	37°	8,000,000
February 17, 1915	12	C	Room	7,700,000
February 18, 1915	12	B	37°	8,600,000
February 21, 1915	12	A	37°	6,200,000
February 27, 1915	12	B	37°	7,300,000
March 2, 1915	12	C	37°	9,200,000
March 11, 1915	48	C	Room	14,600,000
March 11, 1915	12	C	37°	6,300,000
March 28, 1915	9	C	37°	12,900,000
March 30, 1915	12	B	37°	6,800,000
March 30, 1915	12	A	37°	13,000,000
April 19, 1915	24	B	Room	8,100,000
April 19, 1915	24	A	Room	10,900,000
			Average	9,300,000

TABLE XXI—SWEET CURDLERS—NUMBERS OF ORGANISMS IN FIRMLY COAGULATED MILK.

Date	Age of Culture in Hours	Strain	Temperature Held	No. Bact. per c. c.
January 1, 1915	18	B	37°	20,000,000
January 30, 1915	24	B	37°	50,000,000
February 5, 1915	12	C	37°	29,000,000
February 18, 1915	12	C	37°	36,000,000
February 21, 1915	12	A	37°	18,900,000
February 23, 1915	12	C	37°	42,400,000
February, 25, 1915	12	C	37°	45,000,000
February, 25, 1915	12	C	37°	29,200,000
February 26, 1915	12	C	37°	56,000,000
March 2, 1915	12	B	37°	28,400,000
March 5, 1915	12	A	37°	23,900,000
March 6, 1915	12	A	37°	36,200,000
March 8, 1915	12	A	37°	33,700,000
March 8, 1915	9	C	37°	18,400,000
March 10, 1915	9	C	37°	18,200,000
March 18, 1915	9	C	37°	20,200,000
March 19, 1915	9	A	37°	15,950,000
March 20, 1915	9	A	37°	23,400,000
March 22, 1915	24	C	37°	15,950,000
March 23, 1915	24	B	Room	25,600,000
March 23, 1915	24	C	Room	22,200,000
March 23, 1915	24	A	Room	25,600,000
March 24, 1915	24	C	Room	33,000,000
March 24, 1915	9	B	Room	41,200,000
March 26, 1915	9	B	37°	17,000,000
March 31, 1915	18	C	37°	16,700,000
			Average	28,500,000

TABLE XXII—SWET CURDLERS—NUMBER OF ORGANISMS AT TWO-HOUR PERIODS.

Age of Culture in Hours	Strain A. Bacteria per c. c.	Strain A. Condition of Milk	Strain B. Bacteria per c. c.	Strain B. Condition of Milk	Strain C. Bacteria per c. c.	Strain C. Condition of Milk
2	1,000.000	no change	950.000	no change	101.000	no change
4	4,350.000	no change	1,900.000	no change	6,800.000	no change
6	7,700.000	no change	9,600.000	no change	24,300.000	med. coag.
8	8,400.000	slightly coag.	10,200.000	slightly coag.	30,000.000	firmly curdled
10	9,500.000	medium coag.	15,000.000	medium coag.		

data obtained on the milk classed as firmly coagulated. Five of the counts were made on milk held at room temperature while the other 21 were made on milk held at 37° C. The average of the 26 counts is 28,500,000 per c. c. while the individual counts range from 15,950,000 to 56,000,000. All of the room temperature cultures were 24 hours old while the ages of the 37° C. cultures range from 9 to 24 hours.

Table XXII shows the number of organisms found in inoculated milk at two hour intervals up until the time of coagulation. Counts were secured on unchanged milk (up to 9,600,000 per c. c.) which are much larger than the counts obtained on slightly coagulated milk as reported in table XIV.

The counts reported in table XXII are in general fairly high for the various stages of coagulation; these high counts both in unchanged and coagulated milk were secured on cultures considerably younger than the cultures dealt with in tables XIX, XX, and XXI and it seems probable that the age of the culture is important from the standpoint of the changes produced by a given number of organisms. The amounts of the various products secreted by a given organism may depend on the time that elapses before that organism divides and thus the changes produced by a given number of cells may be materially influenced by the rapidity with which division is taking place.

The results obtained on the sweet curdlers indicate that many organisms (in the cases studied always over 1,000,000 per c. c.) are present in milk before there is any evidence of coagulation. The numbers of sweet curdling organisms required to produce changes in milk, however, are considerably smaller than the numbers of organisms required to produce changes in the cases of the other types considered thus far. Moreover the number of sweet curdlers required to bring about *marked* changes in milk is smaller than in the cases of the other types studied. The comparatively small number of sweet curdlers required to produce changes in milk may be due to the comparatively large size of the type; there is at least the possibility that the larger cells may produce products responsible for the changes in milk in larger amounts than the smaller cells.

Organism 736

Organism 736 was isolated from a sample of milk brot to the laboratory from one of the smaller cities of the state. It causes a disagreeable putrefactive odor in milk, later a clearing of the milk, beginning at the surface, and finally practically complete digestion. The organism grows very well at room temperature. Since the original isolation, the organism has been en-

countered a number of times and its description is soon to be published.

SPECIAL METHODS

The inoculated milk was always held at room temperature. Both sterile milk (8 to 15 c. c. in tubes) and aseptic milk (30 to 50 c. c. in tubes) were employed; the aseptic milk was used only for the work on odor while the sterile milk was used for the work on odor and the different stages of digestion. The stages in the development of the cultures have been classed as follows:

(A) Faint Odor: Milk showing a distinct but faint odor.

(B) Marked Odor: Milk showing a pronounced odor.

(C) Beginning Digestion: Milk showing a definite clearing at the surface.

(D) Considerable Digestion: Milk showing a clear layer from 1-8 to 3-8 inches thick at the surface.

(E) Complete Digestion: Milk showing practically complete digestion.

RESULTS OBTAINED

Table XXIII shows the results obtained on milk classed as showing a faint odor. The average for the 20 determinations is 32,000,000 per c. c. and the individual counts vary from 14,000,000 to 57,000,000 while the ages vary from 9 to 42 hours. The data obtained on the milk classed as showing a marked odor is presented in table XXIV; the average of the 37 determinations is 95,000,000 per c. c. while the individual counts range from 66,000,000 to 150,000,000; the ages of the cultures vary from 9 to 24 hours. The data secured on the milk classed as showing beginning digestion is shown in table XXV; the average for the 16 determinations is 202,000,000 per c. c. while the range is from 160,000,000 to 240,000,000. The ages of the cultures vary from 10 to 24 hours. Table XXVI presents the results obtained on the milk classed as showing considerable digestion; the 16 counts range from 250,000,000 to 445,000,000 per c. c. and averages 312,000,000. Most of the cultures were 24 hours old altho one culture of 18 and one of 72 hours are included. The data obtained on milk showing complete digestion is given in table XXVII where the 7 counts range from 470,000,000 to 750,000,000 per c. c. and average 646,000,000; the ages of the cultures vary from 18 to 48 hours.

The data obtained relative to the development of an odor in aseptic milk is shown in table XXVIII. The cultures were not grouped on the basis of the extent of the odor but all were plated when the characteristic odor was definitely present. The 31 counts obtained vary from 19,000,000 to 98,000,000 per c. c. and average 48,000,000. The determinations made on aseptic milk

TABLE XXIII—Organism 736.
Number of Organisms in Milk Showing Faint Odor.

Date	Age of Culture in Hours	No. Bact. per c. c.
Nov. 2, 1914..	24	42,000,000
Jan. 16, 1915..	12	39,000,000
Jan. 28, 1915..	10	22,000,000
Feb. 3, 1915..	12	55,000,000
Feb. 6, 1915..	12	33,000,000
Feb. 6, 1915..	12	39,000,000
Feb. 12, 1915..	36	14,000,000
Feb. 12, 1915..	12	28,000,000
Feb. 15, 1915..	42	29,000,000
Feb. 17, 1915..	12	18,000,000
Feb. 18, 1915..	12	45,000,000
Feb. 25, 1915..	12	24,000,000
March 5, 1915	12	41,000,000
March 6, 1915	12	49,000,000
March 8, 1915	9	25,000,000
March 23, 1915	9	23,000,000
March 24, 1915	9	21,000,000
March 24, 1915	9	19,000,000
March 27, 1915	24	57,000,000
March 28, 1915	12	22,000,000
	Average	32,000,000

TABLE XXIV—Organism 736.
Number of Organisms in Milk Showing Marked Odor.

Oct. 23, 1914..	24	135,000,000
Nov. 1, 1914..	24	82,000,000
Nov. 11, 1914..	24	103,000,000
Nov. 23, 1914..	24	75,000,000
Dec. 2, 1914..	24	120,000,000
Dec. 17, 1914..	18	79,000,000
Dec. 18, 1914..	18	92,000,000
Dec. 19, 1914..	18	135,000,000
Dec. 21, 1914..	18	87,000,000
Dec. 23, 1914..	24	101,000,000
Jan. 9, 1915..	18	104,000,000
Jan. 11, 1915..	24	70,000,000
Jan. 13, 1915..	18	130,000,000
Jan. 15, 1915..	12	120,000,000
Jan. 15, 1915..	12	90,000,000

TABLE XXIV—Continued.

Oct. 31, 1914..	24	228,000,000
Nov. 9, 1914..	24	190,000,000
Nov. 11, 1914..	24	210,000,000
Nov. 13, 1914..	24	220,000,000
Nov. 25, 1914..	24	185,000,000
Dec. 7, 1914..	24	210,000,000
Dec. 11, 1914..	24	210,000,000
Dec. 31, 1914..	24	230,000,000
Jan. 1, 1915..	18	180,000,000
Jan. 8, 1915..	18	180,000,000
Jan. 28, 1915..	10	210,000,000
Jan. 31, 1915..	12	170,000,000
Feb. 10, 1915..	24	231,000,000
Feb. 21, 1915..	12	240,000,000
Feb. 22, 1915..	12	160,000,000
Feb. 24, 1915..	12	172,000,000
	Average	202,000,000

TABLE XXV—Organism 736.
Number of Organisms in Milk Showing Beginning Digestion.

Date	Age of Culture in Hours	No. Bact. per c. c.
Jan. 17, 1915..	12	107,000,000
Jan. 18, 1915..	12	150,000,000
Jan. 19, 1915..	12	76,000,000
Jan. 20, 1914..	12	75,000,000
Jan. 21, 1915..	12	78,000,000
Jan. 22, 1915..	12	77,000,000
Jan. 23, 1915..	12	77,000,000
Jan. 25, 1915..	12	99,000,000
Feb. 2, 1915..	12	72,000,000
Feb. 13, 1915..	12	75,000,000
Feb. 22, 1915..	12	125,000,000
Feb. 26, 1915..	12	66,000,000
Feb. 27, 1915..	12	98,000,000
March 2, 1915	12	88,000,000
March 3, 1915	12	90,000,000
March 8, 1915	9	66,000,000
March 11, 1915	9	79,000,000
March 15, 1915	9	100,000,000
March 18, 1915	10	75,000,000
March 19, 1915	9	116,000,000
March 20, 1915	9	108,000,000
March 26, 1915	9	107,000,000
	Average	95,000,000

show approximately the same numbers as the determinations made on sterile milk showing either a slight odor or a marked odor. As already stated, cows were selected for the production of the aseptic milk on the basis of the low bacterial content of their milk; some of these samples of aseptic milk had, however, a pronounced odor that of course tended to mask the odor produced by the organism inoculated.

If the data obtained with the milk from each animal is considered, a wide variation is noticed; with the milk from cow 69 the 7 determinations range from 25,000,000 to 98,000,000 per c. c., with the milk from cow 207 the 5 counts range from 19,000,000 to 82,000,000 per c. c., with the milk from cow 87 the 6 counts range from 33,000,000 to 70,000,000 per c. c., with the milk from cow 217 the 7 counts range from 30,000,000 to 77,000,000 per c. c., with the milk from cow 143 the 4 counts range from 20,000,000 to 44,000,000 per c. c. and the two counts on the milk from cow 162 are 53,500,000 and 42,500,000 per c. c. Three trials made on

TABLE XXVI—Organism 736.
Number of Organisms in Milk Showing Medium Digestion.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 10, 1914..	24	283,000,000
Oct. 17, 1914..	24	340,000,000
Oct. 29, 1914..	24	445,000,000
Nov. 7, 1914..	24	285,000,000
Nov. 6, 1914..	18	250,500,000
Nov. 18, 1914..	72	440,000,000
Nov. 19, 1914..	24	265,000,000
Nov. 20, 1914..	24	275,000,000
Nov. 22, 1914..	24	290,000,000
Nov. 27, 1914..	24	300,000,000
Dec. 9, 1914..	24	260,000,000
Dec. 13, 1914..	24	250,000,000
Jan. 2, 1915..	24	416,000,000
Jan. 7, 1915..	24	290,000,000
Feb. 3, 1915..	24	290,000,000
Feb. 9, 1915..	24	310,000,000
	Average	312,000,000

TABLE XXVII—Organism 736.
Number of Organisms in Milk Showing Complete Digestion.

Date	Age of Culture in Hours	No. Bact. per c. c.
Oct. 12, 1914..	24	750,000,000
Oct. 14, 1914..	48	710,000,000
Oct. 30, 1914..	24	670,000,000
Nov. 5, 1914..	18	690,000,000
Nov. 12, 1914..	48	490,000,000
Nov. 26, 1914..	24	470,000,000
Nov. 28, 1914..	48	740,000,000
	Average	646,000,000

TABLE XXVIII—Organism 736.
Number of Organisms in Milk Showing Characteristic Odor.

Date	Cow Number	Age of Culture in Hours	No. Bact. per c. c.
1914			
June 10.	69	18	82,000,000
June 10.	69	18	92,500,000
June 10.	69	22	98,000,000
June 10.	213	18	31,500,000
June 10.	213	22	43,000,000
June 10.	87	22	70,000,000
June 15.	87	12	64,000,000
June 15.	207	12	61,000,000
June 15.	213	12	30,000,000
June 15.	69	12	47,000,000
June 15.	87	12	50,000,000
June 15.	207	12	25,000,000
June 16.	213	12	31,000,000
June 16.	69	12	25,000,000
June 16.	162	12	53,500,000
June 16.	143	12	30,000,000
June 16.	207	15	22,000,000
June 17.	87	15	51,500,000
June 17.	143	15	22,000,000
June 17.	213	15	55,000,000
June 17.	162	15	42,500,000
June 19.	69	15	30,000,000
June 19.	87	15	33,000,000
June 19.	207	15	19,000,000
June 19.	213	15	30,000,000
June 19.	143	15	20,000,000
June 22.	143	15	44,000,000
June 22.	213	15	77,000,000
June 22.	207	18	82,000,000
June 22.	87	15	33,000,000
June 22.	69	15	95,000,000
		Average	48,000,000

the same day with milk from cow 69 range from 82,000,000 to 98,000,000 per c. c., a reasonably close agreement. The odor of the freshly drawn aseptic milk varied from day to day and this is undoubtedly in part the cause of the variations in the counts obtained on milk from the same animal. The original odor of the milk is likewise responsible for some of the variation in the data presented in table XXVIII.

With Organism 736 a large number of organisms were present in milk before a change in the odor could be detected. The smallest number of organisms producing an odor in sterile milk was 14,000,000 per c. c. and in aseptic milk 19,000,000 per c. c. From these comparatively low values, there was an increase in the number of organisms with an increase in the changes produced in the milk, until in milk which had recently become completely digested numbers as high as 750,000,000 per c. c. were present. As in the case of the other organisms studied, large variations were encountered in the numbers of organisms present in milk which was classed as showing the same change.

Bact. lactis acidi.

Three strains of *Bacti. lactis acidi* were isolated for use in the

work herein reported; A, came from a good starter; B, from an old discarded starter, and C, from milk that was allowed to sour at room temperature. All the strains curdled milk rapidly and produced the usual color changes in litmus milk. The work with *Bact. lactis acidi* dealt with the numbers of organisms present at various stages in the reduction of litmus milk, the number present in coagulated milk, and the number present in aseptic milk at the time an acid taste was first detected.

SPECIAL METHODS

The work on the reduction of litmus milk and the coagulation of milk was done with tubes (8 to 15 c. c. of milk) for the most part. In the work on the numbers of organisms present in inoculated aseptic milk when an acid taste could first be detected, large test tubes were employed, the milk being drawn directly into sterilized tubes in amounts varying from 30 to 50 c. c. It was customary to compare the flavor of the inoculated aseptic milk, after the various periods of holding, with the flavor of the uninoculated controls. With this method, it was a common occurrence to find a distinct rise in acidity in a sample which could not be classed as sour; accordingly in the tables dealing with the development of an acid taste in aseptic milk, the change in flavor if given. All the inoculated milk was held at room temperature. The following classification of the changes was employed:

(A) Slight Reduction: Litmus milk showing a slight but definite decrease in the intensity of the color.

(B) Medium Reduction: Litmus milk from which the color of the litmus had largely disappeared.

(C) Complete Reduction: Litmus milk from which the color of the litmus had entirely disappeared, except at the very top of the tube.

(D) Coagulation: Milk showing a complete coagulation.

RESULTS OBTAINED

The data obtained on the samples of milk classed as slightly reduced are presented in table XXIX. The average for the 15 determinations is 117,000,000 per c. c. and the individual determinations range from 62,000,000 to 181,000,000 while the ages of the cultures vary from 9 to 24 hours. Table XXX shows the data obtained on milk that was classed as showing medium reduction; the range of the 29 determinations is from 107,000,000 to 300,000,000 per c. c. and the average is 212,000,000 while the ages of the cultures vary from 10 to 24 hours. The results on litmus milk showing complete reduction are shown in table XXXI. The 21 individual determinations range from 340,000,000 to 950,000,-

000 per c. c. and average 560,000,000 while the ages of the cultures vary from 12 to 24 hours. Table XXXII presents the data obtained on tubes showing coagulation. The six determinations range from 1,060,000,000 to 2,890,000,000 per c. c. and average 1,800,000,000, while the ages of the cultures vary from 24 to 48 hours.

Table XXXIII shows the data obtained at two hour intervals from inoculation until complete reduction. From this data, it is evident that large numbers of organisms (as high as 26,900,000 per c. c.) may be present without any change in the litmus. Considerable variation is evident in the data secured in the different trials; inasmuch however as there are no consistent differences between the strains in the preceding tables dealing wtih *Bact. lactis acidi*, it seems probable that this variation cannot be ascribed to the strains used.

The data obtained on aseptic milk are shown in tables XXXIV

TABLE XXIX—Bact. lactis acidi. Number of Organisms in Slightly Reduced Litmus Milk.

Date	Age of Culture in Hours	Strain	No. Bact. per c. c.
Nov. 23-14	24	A	88,000,000
Jan. 30-15	10	C	62,000,000
Jan. 31-15	12	B	100,000,000
Feb. 9-15	12	B	85,000,000
Mar. 18-15	9	C	142,000,000
Mar. 19-15	9	B	155,000,000
Mar. 20-15	9	C	99,000,000
Mar. 21-15	9	A	128,000,000
Mar. 22-15	9	B	143,000,000
Mar. 23-15	9	B	132,000,000
Mar. 24-15	9	C	90,000,000
Mar. 26-15	9	A	181,000,000
Mar. 30-15	9	C	100,000,000
April 2-15	9	A	136,000,000
April 3-15	9	A	116,000,000
		Average	117,000,000

TABLE XXX—Bact. lactis acidi. Number of Organisms in Medium Reduced Litmus Milk.

Nov.	21-14	24	C	240,000,000
Dec.	28-14	18	B	199,000,000
Dec.	19-14	18	B	280,000,000
Dec.	19-14	18	C	290,000,000
Dec.	21-14	18	B	230,000,000
Dec.	31-14	18	B	155,000,000
Dec.	31-14	18	A	230,000,000
Jan.	9-15	18	A	107,000,000
Dec.	3-14	15	B	300,000,000
Dec.	3-14	15	C	145,000,000
Dec.	5-14	15	C	115,000,000
Jan.	15-15	12	C	295,000,000
Jan.	18-15	12	A	290,000,000
Jan.	19-15	12	A	260,000,000
Jan.	20-15	12	A	225,000,000
Jan.	22-15	12	A	240,000,000
Jan.	22-15	12	B	240,000,000
Jan.	23-15	12	C	208,000,000
Jan.	25-15	12	B	128,000,000
Jan.	25-15	12	C	247,000,000
Jan.	28-16	12	A	130,000,000
Feb.	4-15	12	C	255,000,000
Feb.	12-15	12	B	144,000,000

TABLE XXXI—Bact. lactis acidi. Number of Organisms in Completely Reduced Litmus Milk.

Date	Age of Culture in Hours	Strain	No. Bact. per c. c.
Feb. 18-15	12	A	187,000,000
Feb. 25-15	12	A	110,000,000
Feb. 26-15	12	C	242,000,000
Mar. 4-15	12	A	240,000,000
Mar. 5-15	12	A	186,000,000
Mar. 18-15	10	A	239,000,000
		Average	212,000,000
Nov. 27-14	24	A	640,000,000
Nov. 27-14	24	B	800,000,000
Dec. 2-14	24	A	950,000,000
Dec. 5-14	15	A	585,000,000
Dec. 7-14	15	B	580,000,000
Dec. 11-14	18	A	670,000,000
Dec. 12-14	18	B	490,000,000
Dec. 14-14	15	A	388,000,000
Dec. 17-14	18	A	340,000,000
Dec. 18-14	18	B	528,000,000
Dec. 19-14	18	A	375,000,000
Dec. 21-14	18	A	630,000,000
Jan. 2-15	24	A	680,000,000
Jan. 14-15	24	A	515,000,000
Jan. 19-15	12	C	410,000,000
Feb. 5-15	12	A	580,000,000
Feb. 10-15	12	B	480,000,000
Feb. 17-15	12	A	430,000,000
Feb. 18-15	12	C	430,000,000
Feb. 21-15	12	A	740,000,000
Feb. 22-16	12	A	510,000,000
		Average	560,000,000

TABLE XXXII—Bact. lactis acidi. Numbers of Organisms in Litmus Milk Showing Coagulation.

Nov.	25-14	24	A	1,650,000,000
Nov.	29-14	48	A	1,060,000,000
Nov.	30-14	24	A	2,630,000,000
Dec.	6-14	24	A	2,890,000,000
Jan.	7-15	24	A	1,180,000,000
Mar.	3-15	36	A	1,420,000,000
			Average	1,800,000,000

TABLE XXXIII—BACT. LACTIS ACIDI.
Number of Organisms and Acidities at 2 Hour Periods

Age of Culture in Hours	STRAIN A.			STRAIN B.		
	Acidity	Bacteria per c. c.	Cond of Milk	Acidit.	Bacteria per c. c.	Cond of Milk
2	.16	1,750,000	no change	.16	830,000	no change
4	.16	3,200,000	no change	.16	3,900,000	no change
6	.16	15,500,000	no change	.16	26,900,000	no change
8	.18	130,000,000	slight reduction	.18	116,000,000	slight reduction
10	.19	215,000,000	medium reduction	.20	175,000,000	medium reduction
12	.21	365,000,000	complete reduction	.22	250,000,000	complete reduction
14	.25	500,000,000	complete reduction	.24	460,000,000	complete reduction

STRAIN C.

Acidity	Bacteria per c. c.	Cond of Milk
.19	450,000	no change
.19	920,000	no change
.22	8,600,000	no change
.30	52,000,000	slight reduction
.33	125,000,000	medium reduction
.35	300,000,000	complete reduction

to XXIX. The tables show the acidities, and whether there was a sour taste or simply a distinct rise in acidity, as well as the numbers of bacteria present.

Table XXXIV deals with milk from cow 213. The seven samples showing a definite rise in acidity showed bacterial counts ranging from 30,000,000 to 90,000,000 per c. c. and averaging 56,000,000 while the four samples classed as sour showed counts ranging from 53,000,000 to 166,500,000 per c. c. and averaging 104,000,000.

Table XXXV presents the data obtained on milk from cow 69. The 5 samples showing a distinct rise in acidity showed counts varying from 49,000,000 to 112,000,000 per c. c. and averaging 70,000,000 while the seven samples called sour showed counts ranging from 93,000,000 to 198,500,000 per c. c. and averaging 152,000,000.

With milk from cow 207 (table XXXVI) the seven samples showing a definite rise in acidity showed counts ranging from 37,500,000 to 127,000,000 and averaging 70,000,000 per c. c. while the counts on the 4 sour samples ranged from 91,000,000 to 235,000,000 and averaged 156,000,000 per c. c.

Table XXXVII presents the data obtained with milk from cow 87. The counts on the seven samples classed as showing a distinct rise in acidity vary from 57,000,000 to 243,000,000 per c. c. and average 132,000,000, while the 3 samples classed as sour vary from 176,000,000 to 251,000,000 per c. c. and average 219,000,000 per c. c.

In table XXXVIII which deals with milk from cow 162, all the seven samples were classed as showing a distinct rise in acidity; the range was from 67,000,000 to 209,000,000 per c. c. and the average was 121,000,000.

The milk from cow 143 is considered in table XXXIX. The four samples classed as showing a distint rise in acidity showed counts ranging from 146,000,000 to 330,000,000 per c. c. and averaging 227,000,000 while the 3 sour samples showed counts ranging from 203,000,000 to 460,000,000 and averaging 305,000,000 per c. c.

Considering tables XXXIV to XXXIX inclusive, there seems to be no very definite relationship between the development of a sour taste and the increase in acidity. In a number of instances milk showing increases of .04 or .05% in the acidity was called sour, while in one instance a sample of milk showing an increase of only .03% was considered sour. On the other hand, certain samples of milk showing acidity increases of .04 or .05% or even more were classed as showing a distinct rise in acidity only, instead of being definitely sour. One sample of milk showing a rise of only .02% was classed as showing a distinct rise in acidity. These variations in the acid taste of samples of milk showing the same general acidity increases are, in part, undoubtedly due to the masking effect of the other flavors in milk as well as to the masking effect of considerable amounts of fat. It is evident, however, that comparatively small amounts of acid may be responsible for an acid flavor in milk, and this tends to explain the association of what are apparently normal acidities with acid flavors.

There seemed to be only a very general relationship between the rise in acidity and the numbers of organisms present. In the case of cow 69, for example, more organisms were present in a sample of milk showing a rise of only .02% in acidity than in another sample showing a rise of .07% and this same general situation was observed with the milk from the other cows.

With *Bact. lactis acidi* large numbers of organisms were present in milk before there was a reduction of the litmus (if litmus was present) or any other detectable change. The smallest number of organisms present in litmus milk showing a slight reduction of the litmus was 62,000,000 per c. c.; in general, with an increase in the extent of the reduction there is an increase in the number of organisms present. The smallest number of organisms present in milk showing coagulation was 1,060,000,000 per c. c.

In aseptic milk showing, to the taste, a definite rise in acidity or a sour taste there were always large numbers of organisms present. In aseptic milk as in sterile milk, wide variations were observed in the result producted by the same general number of organisms.

TABLE XXXIV– COW 213.

Bact. lactis acidi.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 9, 1915	14	.....	.20	44,000,000	d. r.
June 9, 1915	20	.....	.21	51,000,000	d. r.
June 10, 1915	15	.18	.22	115,500,000	sour
June 10, 1915	22	.18	.23	166,500,000	sour
June 15, 1915	12	.18	.22	58,000,000	d. r.
June 16, 1915	12	.19	.25	53,000,000	sour
June 17, 1915	15	.21	.28	82,500,000	sour
June 22, 1915	15	.15	.19	90,000,000	d. r.
June 23, 1915	15	.13	.20	57,500,000	d. r.
June 24, 1915	15	.15	.19	30,000,000	d. r.
June 26, 1915	15	.15	.22	62,000,000	d. r.

d. r.—distinct rise in acidity.

TABLE XXXV—COW 69.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 9, 1915	14	.....	.19	56,000,000	d. r.
June 9, 1915	20	.....	.21	93,000,000	sour
June 10, 1915	22	.15	.23	190,000,000	sour
June 15, 1915	12	.18	.20	71,000,000	d. r.
June 16, 1915	12	.13	.20	62,000,000	d. r.
June 17, 1915	15	.18	.21	159,000,000	sour
June 19, 1915	15	.14	.19	137,000,000	sour
June 19, 1915	15	.15	.20	150,000,000	sour
June 19, 1915	15	.14	.20	137,000,000	sour
June 22, 1915	15	.13	.19	112,000,000	d. r.
June 23, 1915	18	.15	.21	137,500,000	sour
June 24, 1915	18	.15	.20	49,000,000	d. r.
June 26, 1915	15	.15	.21	198,500,000	sour

d. r.—distinct rise in acidity.

TABLE XXXVI—COW 207.

Bact. lactis acidi.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 9, 1915	20	.....	.21	37,500,000	d. r.
June 10, 1915	15	.15	.21	66,500,000	d. r.
June 10, 1915	22	.15	.24	138,500,000	sour
June 15, 1915	12	.19	.23	60,000,000	d. r.
June 16, 1915	12	.18	.22	67,000,000	d. r.
June 17, 1915	15	.18	.26	159,500,000	sour
June 19, 1915	15	.14	.22	91,000,000	sour
June 22, 1915	15	.18	.22	66,000,000	d. r.
June 23, 1915	15	.18	.23	127,000,000	d. r.
June 24, 1915	15	.18	.22	65,000,000	d. r.
June 26, 1915	15	.18	.27	235,000,000	sour

d. r.—distinct rise in acidity.

TABLE XXXVII—COW 87.

Bact. lactis acidi.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 10, 1915	15	.19	.22	243,000,000	d. r.
June 10, 1915	22	.19	.23	251,000,000	sour
June 15, 1915	12	.18	.22	100,500,000	d. r.
June 16, 1915	12	.16	.21	57,000,000	d. r.
June 17, 1915	15	.18	.24	230,000,000	sour
June 19, 1915	15	.14	.22	176,000,000	sour
June 22, 1915	15	.18	.21	98,000,000	d. r.
June 23, 1915	15	15	.22	196,000,000	d. r.
June 23, 1915	15	15	.22	91,000,000	d. r.
June 26, 1915	15	15	.21	226,000,000	d. r.

d. r.—distinct rise in acidity.

TABLE XXXVIII -COW 162.

Bact. lactis acidi.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 16, 1915	12	.17	.23	76,000,000	d. r.
June 17, 1915	15	.16	.21	145,000,000	d. r.
June 19, 1915	15	.16	.20	162,000,000	d. r.
June 22, 1915	15	.15	.20	96,500,000	d. r.
June 23, 1915	15	.15	.20	92,000,000	d. r.
June 24, 1915	15	.14	.20	67,000,000	d. r.
June 26, 1915	15	.15	.20	209,000,000	d. r.

d. r.—distinct rise in acidity.

TABLE XXXIX—COW 143.

Bact. lactis acidi.

Date	Age of Culture	Orig. Acidity	Acidity of Culture	Bacteria per c. c.	Condition of Milk
June 16, 1915	12	.16	.21	203,000,000	sour
June 17, 1915	15	.16	.23	460,000,000	sour
June 19, 1915	15	.14	.20	252,000,000	sour
June 22, 1915	18	.13	.19	146,000,000	d. r.
June 23, 1915	15	.13	.20	173,000,000	d. r.
June 24, 1915	15	.13	.20	330,000,000	d. r.
June 26, 1915	15	.13	.20	260,000,000	d. r.

d. r.—distinct rise in acidity.

SUMMARY

In the work reported, an effort was made to secure information regarding the numbers of bacteria required to produce various changes in milk. While changes in the flavor and odor first attracted attention, other changes were considered because of the difficulties presented by all work dealing with changes in flavor and odor. Sterile milk was used for most of the work, altho some experiments were carried out with aseptic milk.

A summary of the results obtained is presented in table XL.

From the data presented, it appears that changes in milk due to the growth of bacteria therein occur only after large numbers of bacteria are present; the samples of milk which showed changes of one kind or another always contained over 1,000,000 bacteria per c. c. and ordinarily much larger numbers. The sweet curdlers produced changes in milk with the samllest numbers of organisms and here the smallest number observed with slight coagulation was 1,250,000 per c. c. With some organisms, pronounced changes required approximately 1,000,000,000 per c. c. and between this value and the minimum already mentioned wide variations were encountered.

Wide variations apparently exist in the numbers of organisms present in milk showing the same condition. This is evident from the percentage variation between the minimum and maximum and also by the results obtained when freshly inoculated cultures were plated at two hour intervals for considerable periods; the difficulty of classifying the conditions observed in milk are, in part, responsible for the variations obtained.

When *Bact. lactis acidi* was inoculated into aseptic milk, a dis-

TABLE XL—SUMMARY OF THE NUMBERS OF BACTERIA PRESENT IN MILK WHICH HAS UNDERGONE VARIOUS CHANGES.

Organism	Condition	No. of Determinations	Minimum	Maximum	Average	Remarks
Bact. lactis aerogenes...	Slightly Ropy	7	11,000,000	49,000,000	32,200,000	
	Slightly Ropy	37	79,000,000	156,000,000	116,700,000	
	Medium Ropy	36	160,000,000	285,000,000	223,000,000	
	Very Ropy	37	290,000,000	495,000,000	373,000,000	
	Extremely Ropy	9	500,000,000	830,000,000	650,000,000	
	Extremely Ropy	5	2,870,000,000	4,960,000,000	3,800,000,000	
Bact. lactis viscosum....	Slightly Ropy	19	15,500,000	44,000,000	26,000,000	Milk in thin layers
	Very Ropy	4	450,000,000	786,000,000	556,000,000	
Bitter Organism	Very Slight Coagulation....	18	22,000,000	68,000,000	44,000,000	
	Slight Coagulation	27	84,000,000	167,000,000	119,000,000	
	Base Coagulation	26	181,000,000	375,000,000	289,000,000	
	Nearly Com. Coagulation....	13	415,000,000	690,000,000	545,000,000	
	Freshly Coagulated Cultures..	10	710,000,000	1,500,000,000	1,000,000,000	
	Old Coagulated Cultures....	5	2,060,000,000	2,515,000,000	2,300,000,000	
	Bitter Flavor	10	10,000,000	66,000,000	38,900,000	Aseptic Milk
Sweet Curdlers	Slight Coagulation	21	1,250,000	4,500,000	3,100,000	
	Medium Coagulation	22	6,200,000	14,500,000	9,300,000	
	Firm Coagulation	26	15,950,000	55,000,000	28,500,000	
736	Faint Odor	20	14,000,000	57,000,000	32,000,000	
	Marked Odor	37	66,000,000	150,000,000	95,000,000	
	Beginning Digestion	16	160,000,000	240,000,000	202,000,000	
	Considerable Digestion	16	250,000,000	445,000,000	312,000,000	
	Complete Digestion	7	470,000,000	750,000,000	646,000,000	
	Odor	31	19,000,000	98,000,000	48,000,000	Aseptic Milk
Bact. lactis acidi.....	Slight Reduction of Litmus..	15	62,000,000	181,000,000	117,000,000	
	Medium Reduction of Litmus	29	107,000,000	300,000,000	212,000,000	
	Complete Reduction of Litmus	21	340,000,000	950,000,000	560,000,000	
	Coagulation	6	1,060,000,000	2,890,000,000	1,800,000,000	
	d. r.	4	146,000,000	330,000,000	227,000,000	Aseptic Milk Cow No. 143
	Sour	3	203,000,000	460,000,000	305,000,000	Aseptic Milk Cow No. 143
	Sour	3	176,000,000	251,000,000	217,000,000	Aseptic Milk Cow No. 87
	d. r.	7	57,000,000	243,000,000	132,000,000	Aseptic Milk Cow No. 87
	d. r.	7	67,000,000	209,000,000	121,000,000	Aseptic Milk Cow No. 162
	Sour	7	93,000,000	198,500,000	152,000,000	Aseptic Milk Cow No. 69
	d. r.	5	49,000,000	112,000,000	70,000,000	Aseptic Milk Cow No. 69
	Sour	4	91,000,000	235,000,000	156,000,000	Aseptic Milk Cow No. 207
	d. r.	7	37,500,000	127,000,000	70,000,000	Aseptic Milk Cow No. 207
	Sour	4	53,000,000	166,500,000	104,000,000	Aseptic Milk Cow No. 213
	d. r.	7	30,000,000	90,000,000	56,000,000	Aseptic Milk Cow No. 213

d. r.—Distinct use in acidity.

tinct rise in acidity was commonly detectable by the sense of taste before the milk could be classed as sour. There seemed to be no definite relationship between the rise in acidity and the classification of the milk as sour or as showing a distinct rise in acidity. Acidity increases of .03, .04 or .05% (in one case .02) were detected by the sense of taste and this means that quite low acidities (acidities that would be regarded as normal) may be encountered along with acid flavors in the milk.

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Influence of Humus Forming Materials of Different Nitrogen-Carbon Ratios on Bacterial Activities

By P. E. Brown and F. E. Allison

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Influence of Humus Forming Materials of Different Nitrogen-Carbon Ratios on Bacterial Activities

By P. E. Brown and F. E. Allison

The determination of the nitrogen-carbon ratio in soils is now considered of much importance in fertility studies. Not only does it show the organic matter content of soils more accurately than the more or less arbitrary humus determinations, concerning which considerable difference of opinion exists, but it also throws some light on the rate at which decomposition processes are occurring.

When organic matter is applied, the nitrogen-carbon ratio of the soil is modified to a greater or less extent, depending on the ratio of these elements in the materials used. Stewart[1] has shown that the common humus-forming substances have a much wider ratio than soils and hence the effect of turning under corn stover, oats straw or manure in a soil would be to widen the nitrogen-carbon ratio. The same author has also shown that under normal conditions the nitrogen-carbon ratio of the soil has a tendency to become narrower as the age of the organic matter increases. Other investigators have noted the same narrowing of the nitrogen-carbon ratio in decomposing organic matter and have concluded that it is due to the greater ease with which the carbonaceous portion of the organic matter decomposes and disappears than the nitrogenous part. Furthermore, as the more actively decomposable portions of the organic matter are removed, the remainder consists of rather inert materials whose decomposition proceeds more slowly and with much more difficulty.

The presence, therefore, of a narrow nitrogen-carbon ratio in soils might be considered to show a deficiency in fresh organic matter and consequently a lack of the proper decomposition processes for the production of optimum amounts of available plant food. This is actually the case in humid soils. Experience has shown that if the ratio narrows beyond a point of about 1 to 10, crop yields may be reduced, evidently because of an in-

[1] Stewart, Robt., Quantitative relationships of carbon, phosphorus and nitrogen in soils. Bull., Ill. Agr. Expt. Sta. 145. (Stewart gives also a complete bibliography of investigations on nitrogen and carbon in soils.)

sufficient production of available nitrogen, phosphorus and potassium. On the other hand, if the ratio is 1 to 12 or above, bacterial activities apparently occur to a satisfactory extent and enough soluble plant food is produced for good crop growth.

This question now arises: When a soil shows a narrow nitrogen-carbon ratio and hence a lack of fresh organic matter, should materials of the widest possible ratio be chosen to supply the deficiency? In other words, would the bacterial activities and crop yields be benefited as much by additions of straw as by turning under a crop like clover which has a much narrower nitrogen-carbon ratio, but at the same time supplies more nitrogen?

It is commonly believed that clovers or other leguminous green manure crops are more valuable for supplying organic matter than straws or stover, but the latter may be applied more cheaply and if they will serve as well to increase bacterial activities and crop yields they should be used.

Obviously, the nitrogen content of the soil should be considered in choosing materials to increase the organic matter content. When nitrogen is lacking, should leguminous crops be employed because of the nitrogen which they supply? Would it not be quite as satisfactory to increase the organic matter content of the soil and the decomposition processes by using a cheaper material which would increase the fixation of nitrogen from the atmosphere? Would these cheaper materials exert a sufficiently greater effect on bacterial activities, especially on azofication or non-symbiotic nitrogen-fixation, to prove as valuable as the leguminous green manures?

In other words, would straws increase the nitrogen content of the soil sufficiently through azofication to keep the crop as well supplied with nitrogen as when the legumes are used? Again, would the nitrogen in the soil be made available as rapidly by the decomposition produced by the straws, as that element in the legumes is made available by the decomposition which they engender?

These questions arose from a consideration of the nitrogen-carbon ratio in soils and the experiments reported in the following pages were planned to answer them. Briefly, the purpose of this work was to study the influence on certain bacterial activities of materials of narrow and of wide nitrogen-carbon ratio, when applied to soils low in organic matter. The processes studied were those which are important from the standpoint of the decomposition of nitrogenous organic matter, namely, ammonification and nitrification, and that which concerns the increase in soil nitrogen namely, azofication or non-symbiotic nitrogen-fixation.

The comparative effects of these materials on the growth of

oats in greenhouse pots were also studied in the attempt to ascertain whether the crop yields were affected in a similar manner to the bacterial processes. It was also sought to determine whether inexpensive materials of a narrow nitrogen-carbon ratio would not stimulate bacterial activities and, especially, increase the fixation by the soil of nitrogen from the atmosphere sufficiently to give as satisfactory yields as materials of a wider ratio.

THE PLAN OF THE EXPERIMENT

The soil chosen for this work was secured from one of the college experimental orchards and is classed by the Bureau of Soils as Miami sandy loam. It was low in organic matter and slightly acid in reaction, showing a lime requirement (according to the Veitch method) of 736 lbs. of calcium carbonate per acre of 2,000,000 lbs. of surface soil. Before the special treatments were made, therefore, sufficient calcium carbonate was applied to neutralize the acidity and bring the lime content of the soil up to two tons per acre.

After being thoroughly sieved and air-dried and receiving the lime as mentioned, the soil was filled into thirty-six earthenware pots in the greenhouse, at the rate of 36 pounds per pot.

The special treatments of the pots were as follows:

1-2—Check.
3-4—15 tons horse manure per acre.
5-6—15 tons cow manure per acre.
7-8—15 tons rotted manure per acre.
9-10—2½ tons oats straw per acre.
11-12—3 tons corn stover per acre.
13-14—2 tons timothy hay per acre.
15-16—4 tons cowpea hay per acre.
17-18—4 tons clover hay per acre.
19-20—Check.
21-22—15 tons horse manure per acre.
23-24—15 tons cow manure per acre.
25-26—15 tons rotted manure per acre
27-28—2½ tons oats straw per acre.
29-30—3 tons corn stover per acre.
31-32—2 tons timothy hay per acre.
33-34—4 tons cowpea hay per acre.
35-36—4 tons clover hay per acre.

Plots 19 to 36 were seeded to oats and the others were kept bare to allow of the taking of samples for bacteriological tests.

The rate of application of the materials used was based on farm conditions, approximately the same amounts being applied as if a maximum crop were grown and turned under in the soil or a heavy application of manure were made.

All of the materials were dried and ground before being applied, but the application of the manures was calculated on the

wet basis while in all the other cases the dry basis was used. All applications were figured on the basis of 2,000,000 lbs. of soil per acre.

After mixing the materials thoroly with the soil, the oats were seeded and all received 100 c. c. of an infusion made by shaking for five minutes, fresh soil with water in the proportion of 100 grams per 200 c. c. of water. This was to supply a vigorous bacterial flora from the soil in its natural state in order that the decomposition of the various materials might proceed as rapidly as it would in the field. The optimum moisture content of the soil was determined and after the addition of the infusions sufficient additional water was supplied to bring the water content in each pot up to the optimum. The pots were then weighed and additions of water were made at regular intervals during the continuance of the experiment to maintain a constant weight. The oats were harvested just prior to maturity and were dried, ground and analyzed.

Samples were drawn for bacteriological tests once every two or three weeks and the ammonifying, nitrifying, and azofying or nitrogen-fixing powers of the soils were determined. The casein-fresh-soil method [2] and the dried blood-fresh-soil method were used for ammonification. The ammonium-sulfate-fresh-soil method served for nitrification and the mannite-fresh-soil and dextrose-fresh-soil methods were employed for azofication.[3] The samples for the bacteriological tests were drawn with the usual precautions to avoid contamination and thoro mixing was insured before the 100 gram portions were weighed out for the various tests. The moisture content of all the soils was determined at each sampling and the moisture content of the soils in all the tests was adjusted to two-thirds of the saturation point. In the nitrification tests the moisture content of the samples was kept up by additions of sterile water to weight every ten days. The incubation took place at room temperature which was fairly constant at 23-25° C. The incubation period varied as will be noted in the later discussions.

The ammonification determinations were made in all cases except one by the magnesium-oxide method. In one instance the aeration method of Potter and Snyder [4] was used.

The nitrate determinations were made by the phenoldisulfonic acid method and total nitrogen was estimated by the regular Kjeldahl method.

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[2] Brown, P. E., Methods for the bacteriological examination of soils. Rsch. Bull., Iowa Agr. Expt. Sta. 11. 1912.

[3] Lipman, J. G., Suggestions concerning the terminology of soil bacteria. Botan. Gaz., 51: 454. 1911.

[4] Potter, R. S. and Snyder, R. S., The determination of ammonia in soils. Rsch. Bull. Iowa Agr. Expt. Sta. 17; also Journ. Ind. & Eng. Chem. 73: 221. 1915.

THE EFFECT OF THE MATERIALS ADDED ON THE NITROGEN-CARBON RATIO IN THE SOIL

The nitrogen and the carbon content of the soil and of all the materials used was determined and the nitrogen-carbon ratio calculated. These results are given in table I.

TABLE I. NITROGEN AND CAREON IN SOIL AND IN MATERIALS USED

Materials Analyzed	Nitrogen Percent.	Carbon Percent.	Nitrogen-carbon Ratio
Soil	0.0988	1.3481	1 : 13.644
Horse manure	1.6468	38.7614	1 : 23.537
Cow manure	2.4176	36.6160	1 : 15.145
Rotted manure	2.4461	23.9047	1 : 9.772
Oats straw	.8590	38.1622	1 : 44.426
Corn stover	1.4762	39.8266	1 : 26.979
Timothy hay	.9727	38.1502	1 : 39.221
Cowpea hay	2.1852	42.1834	1 : 19.304
Clover hay	2.0564	41.3085	1 : 20.088

The soil used showed a satisfactorily wide ratio and hence the effects of the materials added cannot be expected to appear as definitely as might be the case did the soil itself contain a smaller amount of organic matter of a narrower ratio.

The rotted manure had the narrowest ratio of any of the materials employed and the oats straw the widest. The cow manure had a narrower ratio than the horse manure and the relative amounts of nitrogen and carbon in the legume hays were about the same as those in the horse manure.

TABLE II. NITROGEN CARBON RATIO IN SOILS AFTER TREATMENT

Pot No.	Treatment	Materials Added Gms.	N Added Gms.	C Added Gms.	Total N Gms.	Total C Gms.	N-C Ratio
1	Check	none	none	none	16.14	220.26	1 : 13.6
2	Check	none	none	none	16.14	220.26	1 : 13.6
3	Horse manure	78.19	1.29	30.31	17.43	250.57	1 : 14.4
4	Horse manure	78.19	1.29	30.31	17.43	250.57	1 : 14.4
5	Cow manure	59.63	1.44	21.84	17.59	242.10	1 : 13.8
6	Cow manure	59.63	1.44	21.84	17.59	242.10	1 : 13.8
7	Rotted manure	83.65	2.05	20.00	18.19	240.26	1 : 13.2
8	Rotted manure	83.65	2.05	20.00	18.19	240.26	1 : 13.2
9	Oats straw	40.85	0.35	15.59	16.50	235.85	1 : 14.3
10	Oats straw	40.85	0.35	15.59	16.50	235.85	1 : 14.3
11	Corn stover	49.02	0.72	19.52	16.87	239.79	1 : 14.2
12	Corn stover	49.02	0.72	19.52	16.87	239.79	1 : 14.2
13	Timothy hay	32.68	0.32	12.47	16.46	232.73	1 : 14.1
14	Timothy hay	32.68	0.32	12.47	16.46	232.73	1 : 14.1
15	Cowpea hay	65.36	1.43	27.57	17.57	247.83	1 : 14.1
16	Cowpea hay	65.36	1.43	27.57	17.57	247.83	1 : 14.1
17	Clover hay	65.36	1.34	27.00	17.49	247.26	1 : 14.1
18	Clover hay	65.36	1.34	27.00	17.49	247.26	1 : 14.1

Table II shows the amounts of nitrogen and carbon added to the soils in the various materials applied and the nitrogen-carbon ratio in the soils after the applications were made. All the materials applied widened the nitrogen-carbon ratio except the rotted manure, which narrowed it. This is in accord with the results in table I which showed that the rotted manure had a narrower ratio than the soil itself and hence might be expected to narrow the ratio in the soil. The oats straw widened the ratio more than any of the hays, particularly the legumes, which is what would be expected. The horse manure brought about a greater widening of the ratio than the other materials, greater even than those which themselves had a wider ratio. This is evidently due to the very much larger application of the horse manure than of the straw, stover, and hays.

The amounts of all the materials used were calculated as maximum field applications and hence it is interesting to note the relative influence of the different substances and consider them from the field standpoint. Rotted manure actually narrowed the ratio and may be considered as having the least effect on the decomposition processes; all the other materials increased the proportion of carbon to nitrogen and hence should increase bacterial activities to a much greater extent.

Among the straws and hays used, the wider the nitrogen-carbon ratio, the greater the widening of the ratio in the soil when they were applied. It might be expected, therefore, that the materials of the wider ratios would give greater effects on bacterial processes than those whose content in nitrogen and carbon was more nearly the same.

The changes in the nitrogen-carbon ratio in this soil, by the applications of these materials, were very much smaller, undoubtedly, than would have occurred if a soil of a narrower ratio had been chosen. It is apparent, however, that the ordinary humus-forming materials on the farm widen the nitrogen-carbon ratio of the soil, even when it is not extremely narrow. Hence they should be expected to increase bacterial activities to a beneficial extent, and also, as a consequence, the decomposition processes, the production of available plant food, and the fixation of nitrogen from the atmosphere.

THE AMMONIFICATION EXPERIMENTS

The experiment was started December 5, and the first sampling was made December 24, in order to allow time for decomposition to commence. Samplings were made approximately every two weeks, the dates being January 7, January 28, February 12, February 26, and March 12.

The results of the ammonification tests using casein and those secured with dried blood are considered separately, but general

conclusions will be drawn from both lots of experiments. The incubation period in the case of casein was three and four days, while with the dried blood it was six and seven days.

THE AMMONIFICATION OF CASEIN

The results of the ammonification experiments with casein are given in table III and in table IV, they are reported in a summarized form. Where individual results were widely at variance with the general trend of the results as shown thruout the six samplings, they are omitted from the averages. The tests at the first three samplings were incubated four days and those on the remaining dates were incubated three days.

Very much larger amounts of ammonia were produced in the samples taken on January 7 than in the tests at other dates. This is probably due to an increase in room temperature to 29° C., which occurred while the samples were being incubated.

It will be seen in table III that for the most part the duplicate determinations agreed very satisfactorily, or at least much better than in the case of other tests with different nitrogenous materials.

The differences in ammonifying power between the different soils were not, however, very large, and it is very difficult in such cases to draw definite conclusions.

In general, it appears from table IV that horse manure, cow manure, and rotted manure favored the ammonifying bacteria to the greatest extent. Next in order came clover hay, corn stover, oats straw, cowpea hay, and timothy hay, respectively. In the case of the latter materials the differences were not large, and their relative effects varied greatly at the different samplings.

For the most part, however, all the materials increased the ammonifying power of the soil, according to the tests, and while in a few instances some depressing action was noted, the figures were not widely enough separated for the results to be conclusive.

Some depression in the ammonifying power of soils may occur immediately following the application of materials similar to those used in these experiments, but after such substances commence to decompose, any decrease in the activities of the ammonifying bacteria would hardly be expected. Some decomposition of all the materials used in this work had undoubtedly occurred prior to the making of any tests and hence it seems probable that the slight depressions noted should be considered merely as indications of the absence of any particular increasing action of the substances applied. The variations from the results with the check soils should in such a case be considered as due to experimental error, or accidental contamination. Much more distinctive results than those secured here must be obtained before

TABLE III. THE AMMONIFICATION OF CASEIN

Pot. No.	Lab. No.	I December 24		II January 7		III January 28		IV February 12		V February 26		VI March 12	
		Ammonia Mgs. N.	Av. Mgs. N.	Ammonia Mgs. N.	Av. Mgs. N.	Ammonia Mgs. N.	Av. Mgs. N.	Ammonia Mgs. N.	Av. Mgs. N.	Ammonia Mgs. N.	Av. Mgs. N.	Ammonia Mgs. N.	Av. Mgs. N.
1	1	82.32		100.94		85.63		84.38		88.69		92.39	
	2	92.47	87.54	104.72	102.84	89.29*	85.63	86.15	85.26	90.47	89.58	94.42	93.40
2	3	87.06		101.85		83.67		83.66		88.58		91.44	
	4	90.97	89.01	103.40	102.62	89.15*	83.67	85.39	84.82	90.37	89.47	94.69	93.05
3	5	90.29		105.57		92.05		87.04		92.75		96.31	
	6	90.97	90.63	104.72	105.14	94.22	93.13	85.99	86.51	92.16	92.45	95.50	95.90
4	7	89.76		103.83		90.28		89.89		92.75		98.48	
	8	84.05*	89.76	104.49	104.16	86.61	88.45	85.27	87.58	lost	92.75	94.42	96.45
5	9	86.08*		105.57		86.19		84.91		96.03		98.88	
	10	89.01	89.01	108.68	107.12	89.99	88.09	87.24	86.07	93.38	94.70	93.88	96.38
6	11	88.49		104.72		81.83*		85.09		92.75		98.34	
	12	89.99	89.24	104.49	104.60	85.73	85.73	85.27	85.18	92.75	92.75	94.42	96.38
7	13	88.49		103.83		90.17		85.09		91.26		94.01	
	14	89.01	88.75	105.81	104.82	87.31	88.74	86.87	85.98	92.75	92.00	86.02*	94.01
8	15	89.01		102.93		85.63		86.51		92.45		94.28	
	16	90.97	89.99	109.11	106.02	84.50	85.06	87.60	87.05	93.05	92.75	93.34	93.81
9	17	86.53		106.04		85.63		85.79		91.86		93.74	
	18	90.97	88.75	105.38	105.71	83.38	84.50	82.41	84.10	93.38	92.62	94.55	94.14
10	19	86.51		98.79		81.96		82.60		92.45		92.25	
	20	90.29	88.90	100.76	99.77	83.67	82.81	82.60	82.60	94.54	93.49	88.59	90.42
11	21	89.01		102.51		82.25		87.42		87.98		92.93	
	22	90.97	89.99	100.76	101.63	88.87	85.56	85.09	86.25	90.47	89.22	94.55	93.74
12	23	89.76		105.10		84.80		83.49		88.47		92.79	
	24	90.97	90.36	102.51	103.80	89.43	87.12	86.71	85.10	90.67	89.57	88.32*	92.79
13	25	92.17		102.32		86.61		86.87		88.18		90.90	
	26	88.14	90.25	105.10	103.71	82.58	84.52	84.02	85.44	88.58	88.88	89.81	90.35
14	27	89.54		104.25		84.38		86.71		92.45		93.20	
	28	88.49	89.01	97.70	100.97	78.73	82.55	84.54	85.62	92.16	92.30	89.27	91.23
15	29	85.55		105.10		78.59		85.27		91.56		91.72	
	30	85.55	85.55	99.21	102.25	78.55	78.57	84.45	84.81	88.47	90.01	96.04	93.88
16	31	89.54		105.53		78.59		85.99		91.26		87.78	
	32	89.54	89.54	107.83	106.67	80.14	79.36	88.29	87.14	lost	91.26	91.44	89.61
17	33	90.97		108.03		86.33		87.76		91.26		93.61	
	34	89.99	90.48	106.23	107.13	87.18	86.75	87.60	87.68	88.58	89.92	91.57	92.59
18	35	90.97		105.57		86.47		87.60		88.58		93.88	
	36	87.06	89.01	107.13	106.35	81.83*	86.47	85.09	85.78	88.47	88.52	92.52	93.20

* Results omitted from the averages.

TABLE IV. THE AMMONIFICATION OF CASEIN

Pot No.	Treatment	I		II		III	
		Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.
1	Check	87.54		102.84		85.63	
2	Check	89.01	88.27	102.62	102.73	83.67	84.65
3	Horse manure	90.63		105.14		93.13	
4	Horse manure	89.76	90.19	104.16	104.65	88.45	90.79
5	Cow manure	89.01		107.12		88.09	
6	Cow manure	89.24	88.39	104.60	105.86	85.73	86.91
7	Rotted manure	88.75		104.82		88.74	
8	Rotted manure	89.99	89.37	106.02	105.42	85.06	86.90
9	Oats straw	88.75		105.71		84.50	
10	Oats straw	88.90	88.82	99.77	102.74	82.81	83.65
11	Corn stover	89.99		101.63		85.56	
12	Corn stover	90.36	90.17	103.80	102.71	87.12	86.34
13	Timothy hay	90.25		103.71		84.52	
14	Timothy hay	89.01	89.63	100.97	102.34	82.55	83.53
15	Cowpea hay	85.55		102.25		78.57	
16	Cowpea hay	89.54	87.54	106.67	104.46	79.36	78.96
17	Clover hay	90.48		107.13		86.75	
18	Clover hay	89.01	89.74	106.35	106.74	86.47	86.61

Pot No.	Treatment	IV		V		VI	
		Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.
1	Check	85.26		89.58		93.40	
2	Check	84.82	85.04	89.47	89.52	93.05	93.22
3	Horse manure	86.51		92.45		95.90	
4	Horse manure	87.58	86.54	92.75	92.60	96.45	96.17
5	Cow manure	86.07		94.70		96.38	
6	Cow manure	85.18	85.62	92.75	93.72	96.38	96.38
7	Rotted manure	85.98		92.00		94.01	
8	Rotted manure	87.05	86.56	92.75	92.35	92.79	93.91
9	Oats straw	84.10		92.62		94.14	
10	Oats straw	82.60	83.35	93.49	93.05	90.42	92.28
11	Corn stover	86.25		89.22		93.74	
12	Corn stover	85.10	85.67	89.57	89.38	92.79	93.26
13	Timothy hay	85.44		88.88		90.35	
14	Timothy hay	85.62	85.53	92.30	90.59	91.23	90.79
15	Cowpea hay	84.81		90.01		93.88	
16	Cowpea hay	87.14	85.97	91.26	90.63	89.61	91.74
17	Clover hay	87.68		89.92		92.59	
18	Clover hay	85.78	86.73	88.52	89.22	93.20	92.89

the occurrence of any depressing action could be considered as the rule with the use of these materials.

In short, it seems safe to conclude that applications of humus forming materials increased the ammonifying power of soils as indicated by tests with the casein-fresh-soil method. The manures had a greater effect than straw, stover, or hays, and horse manure and cow manure showed much more influence than rotted manure. It must be recalled here that the bacterial factor was the same in all the pots as the materials were all added in a dry condition and different effects were due, therefore, to differences in amounts added or in composition.

While the casein-fresh-soil method gives very satisfactory results from the standpoint of agreement of duplicates and because of ease of manipulation, it is apparent that some further modification will be necessary to make it possible for distinctive results to be secured with its use. The dried-blood-fresh-soil method, altho much more difficult to use, is evidently better suited for general soil studies and causes a wider difference to be shown in ammonifying power between soils differently treated.

Reference will again be made to the results with casein after the dried-blood experiments are considered.

THE AMMONIFICATION OF DRIED BLOOD

The samples drawn on the same dates as previously mentioned when the ammonifying power of the soils was tested with casein were used for ammonification tests with dried blood, except that no tests were made on January 7. An additional sampling was made, however, on March 24, so that six series of results were secured here. Thus there was provided a comparison of the two methods as well as additional data on the ammonifying power of the soils.

The results of the tests with dried blood are given in table V and the summarized results appear in table VI.

The incubation period for the first, third, and fifth sampling was seven days; for the fourth and sixth it was six days and in the second series one-half of the determinations were made on the fifth day and the duplicate half on the sixth day. This second series was distilled by the aeration method and this fact, together with the shorter incubation period, explains the low results.

It is commonly recognized that the magnesium oxide method for ammonia gives more than just the ammonia present as such in the soil, breaking down as it does certain amino-compounds and liberating ammonia from them. The aeration method liberates only the ammonia present as such in the soil. The manipulation of the aeration method is somewhat difficult, and especially to keep the entire 100 grams of soil used in each test in such motion as is necessary for accurate results, and hence it was not used for the other tests. The results with magnesium oxide may not be absolutely accurate for ammonia as such, therefore, but they are comparative at least, which, after all, is the main consideration in the ammonification studies reported in this work.

The duplicate determinations, as is usually the case with dried blood, did not agree very closely. This is the chief objection to the dried blood method and is due partly to the lack of uniformity in the composition of the dried blood and the difficulty of thoro mixing with the soil, and largely to the difficulty in dis-

TABLE V. THE AMMONIFICATION OF DRIED BLOOD

Pot. No.	Lab. No.	I December 24		II January 28		III February 12		IV February 26		V March 12		VI March 24	
		Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Ngs. N.
1	1	75.31		29.09		lost		95.33*		52.57		57.73	
	2	78.83	77.07	67.71*	29.09	lost	.....	89.07*		41.98	47.27	32.56*	57.73
2	3	87.05		25.90		lost		46.88	.....	43.87		67.71	
	4	59.47	73.28	32.22	29.06	42.15	42.15	51.86	49.37	42.60	43.73	93.54*	67.71
3	5	92.63		25.11*		lost		58.31		57.19		91.17	
	6	96.24	94.43	50.95	50.95	62.52	62.52	91.71	75.01	73.71	65.45	93.74	92.45
4	7	102.90		37.70		lost		81.45		76.62		87.67	
	8	86.26	94.58	lost	37.70	78.36	78.36	81.16	81.30	97.96	87.29	113.09	100.38
5	9	116.58		33.55		lost		101.38		76.62		68.93	
	10	97.00	106.79	lost	33.55	52.34	52.34	89.37	95.36	71.18	73.90	83.95	76.44
6	11	93.12		47.08		lost		101.67		92.38		46.77*	
	12	70.43	81.77	72.31	59.69	75.53	75.53	108.12	104.89	74.58	83.48	100.39	100.39
7	13	83.44		44.71		lost		96.98		46.72		70.65	
	14	129.67	111.55	57.22	50.96	90.81	90.81	109.88	103.43	54.75	50.73	88.65	79.65
8	15	110.91		39.13		lost		105.48		95.92		111.74	
	16	102.12	106.51	73.13	56.13	73.21	73.21	119.25	112.36	73.90	84.91	88.04	99.89
9	17	78.63		44.04		lost		62.70		63.03		53.39	
	18	89.57	84.10	60.28	52.16	97.60	97.60	55.67	59.18	58.14	60.58	76.52	64.95
10	19	87.04		43.70		lost		78.23		55.15		81.66	
	20	84.16	85.60	40.32	42.01	51.20	51.20	87.31	82.77	94.97	74.56	91.39	86.44
11	21	83.91		lost		lost		91.12		88.44		103.33	
	22	87.25	85.58	55.53	51.81	43.85	43.85	74.42	82.77	82.33	85.38	64.17	83.75
12	23	83.57		43.01		lost		79.99		66.97		78.87	
	24	84.29	83.43	59.26	51.13	68.74	68.74	66.80	73.38	81.14	74.07	51.25	65.06
13	25	112.09		52.85		lost		75.01		143.68		87.67	
	26	76.43	94.36	75.39	64.12	63.65	63.65	110.17	92.59	69.65	106.36	91.39	89.53
14	27	102.71		46.41		lost		108.41		152.72		83.95	
	28	107.40	105.05	73.43	59.92	67.05	67.05	132.14	120.27	51.07	101.89	87.08	85.51
15	29	88.60		33.91		lost		66.51		94.56		68.10	
	30	61.43	75.01	53.84	43.87	61.39	61.39	77.35	71.93	102.03	98.29	70.67	69.37
16	31	85.68		42.01		lost		77.65		122.01*		73.67	
	32	84.81	85.24	72.91	57.46	51.77	51.74	67.98	72.81	81.78	81.78	72.99	73.48
17	33	83.90		51.16		lost		79.40		52.76		65.17	
	34	78.84	81.37	50.14	50.65	57.43	57.43	89.37	84.38	58.82	55.76	62.82	63.99
18	35	92.53		40.00		lost		66.80		129.75		62.43	
	36	81.75	87.14	62.29	51.14	107.22*		67.68	67.24	106.11	117.93	88.85	75.64

* Results omitted from the averages.

TABLE VI. THE AMMONIFICATION OF DRIED BLOOD

Pot No.	Treatment	I		II		III	
		Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.
1	Check	77.07		28.09		.....	
2	Check	73.28	75.14	29.06	29.07	42.15	42.15
3	Horse manure	94.43		50.95		62.52	
4	Horse manure	94.58	94.50	37.70	44.32	78.36	70.44
5	Cow manure	106.71		33.55		52.34	
6	Cow manure	81.77	94.28	59.69	46.62	75.53	63.43
7	Rotted manure	111.55		50.96		90.81	
8	Rotted manure	106.51	109.00	56.13	53.54	73.21	82.01
9	Oats straw	84.10		52.16		97.60	
10	Oats straw	85.69	84.85	42.01	47.08	51.20	74.40
11	Corn stover	85.58		51.81		43.85	
12	Corn stover	83.43	84.50	51.13	51.47	68.74	56.29
13	Timothy hay	94.36		64.12		62.65	
14	Timothy hay	105.05	99.70	59.62	62.02	67.05	65.35
15	Cowpea hay	75.01		43.87		61.39	
16	Cowpea hay	85.24	80.12	57.46	50.16	51.77	56.58
17	Clover hay	81.37		50.65		57.43	
18	Clover hay	87.14	84.25	51.14	80.89	.....	57.43

Pot No.	Treatment	IV		V		VI	
		Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.	Ammonia Mgs. N.	Average Mgs. N.
1	Check	.....		47.27		57.73	
2	Check	49.37	49.37	43.73	45.50	67.71	62.72
3	Horse manure	75.01		65.45		92.45	
4	Horse manure	81.30	78.15	87.29	76.37	100.38	96.41
5	Cow manure	95.36		73.90		76.44	
6	Cow manure	104.89	100.13	83.48	78.69	100.39	88.41
7	Rotted manure	103.43		50.73		79.65	
8	Rotted manure	112.36	107.89	84.91	67.82	99.89	89.77
9	Oats straw	59.18		60.58		64.95	
10	Oats straw	82.77	70.97	74.56	67.57	86.41	75.72
11	Corn stover	82.77		85.38		83.75	
12	Corn stover	73.38	78.07	74.07	79.72	65.06	74.40
13	Timothy hay	92.59		106.36		89.53	
14	Timothy hay	120.27	106.43	101.89	104.12	85.51	87.52
15	Cowpea hay	71.93		98.29		69.37	
16	Cowpea hay	72.81	72.37	81.78	90.03	73.48	71.42
17	Clover hay	84.38		55.76		63.99	
18	Clover hay	67.24	75.81	117.93	86.84	75.64	69.81

tilling because of foaming. In some instances where the results were clearly abnormal they were not included in the averages.

Considering now the results in table VI, it is apparent that the different materials affected quite differently the ammonifying power of the soils as determined by the dried blood method.

The effects of treatment were much more pronounced than with the casein method. There was in no case a decrease in ammonifying power, and hence the few slight depressions noted with the casein were evidently due to an absence of indications of effect rather than to an actual depression of ammonifying power.

Again, it appears that the manures favored the ammonifying

process very largely. With only one exception the manures all showed greater effects on the ammonifying bacteria than any of the other materials. The rotted manure gave a greater influence than the cow or horse manure. It will be recalled that the rotted manure had the narrowest ratio of any of the materials and it gives here the greatest effect on the ammonifying power of the soil.

The general influence of the horse and cow manure was about the same, the horse manure having a slight advantage. Their nitrogen-carbon ratios were quite different, that of cow manure being much narrower than that of horse manure, so it appears here that the difference in ratio had little effect on the influence exerted by the materials on ammonification.

The most surprising results were those from the soils treated with timothy hay, which were about as high as those from the soils receiving rotted manure, and higher in almost every case than those from the soils where horse and cow manure were used. The timothy hay had a wide nitrogen-carbon ratio, very much wider than the other materials except the oats straw. It might seem, therefore, that materials of wide nitrogen-carbon ratio would exert as much effect on ammonification as those of a narrow ratio. When the results with oats straw are considered, however, it is found that the effects on the ammonifying bacteria were much less than the influence exerted by the manures and even less than the effect of the hays which had a narrower nitrogen-carbon ratio.

Practically the same situation obtained in the case of the corn stover, and the effect on the ammonifying bacteria was less than that exerted by the manures and hays, which had narrower ratios of nitrogen and carbon. It seems, therefore, that the nitrogen-carbon ratio in the common humus-forming materials was of very little influence on the extent of the action exerted on the ammonifying becteria.

The cowpea hay and clover hay uniformly exerted less influence on the ammonifying process than the manures and the timothy hay but they showed more effect than the oats straw and corn stover, altho the differences were not very great and there were some variations in effects shown at the different samplings.

It is apparent, therefore, that some other factors than the nitrogen-carbon ratio in the materials used in this work must be of more importance in determining the effect on the ammonifying bacteria. It is probable that the character of the chemical compounds present in the materials used would explain the variations noted, for the different substances are made up, of course, of very different chemical substances, and while these differences would not appear from the analyses for carbon and nitrogen,

they are undoubtedly present and of much significance from the standpoint of effects on bacterial activities.

It will be recalled that the materials were all applied dry so that the variations noted were not due to the bacterial content of the substances. Of course, different quantities were used, amounts such as are common on the farm being employed. These differences in applications probably account partially for some of the results noted, such, for instance, as the greater effect of the manures, but inasmuch as the results are to be interpreted from the field standpoint it was necessary to make field applications and these differences in amounts are inherent in farm practice.

In general, therefore, it appears from these results that applications of the common humus-forming materials in maximum amounts employed on the farm, led to increases in the ammonifying power of the soil. Furthermore, these increases were apparently independent of the nitrogen-carbon ratio of the materials added and probably dependent on the chemical composition of the substances. Manures gave the greatest effects in most cases, altho the timothy hay used gave a greater influence than the horse or cow manure. In the field, under ordinary farm conditions, when the manures are applied in a fresh condition and teeming with bacteria, a greater effect of the manures on ammonification would be expected.

Similarly, while the rotted manure gave the greatest influence in these results, in the field, it is probable that the fresh manures would show more effect because of the actual bacteria added.

If the soil employed had possessed a narrower nitrogen-carbon ratio, the differences in the results might have been more pronounced, so that any conclusion from this work must be qualified by specifying *for this particular soil which possessed a satisfactorily wide nitrogen-carbon ratio.* With this qualification, then, the statement previously made may be accepted as a rather definite conclusion from these results, namely, that the nitrogen-carbon ratio of common humus-forming materials, used in maximum field applications, had little or no effect on the influence exerted by these substances on ammonification in *this particular soil.* The different effects were probably due to the variations in chemical composition of the materials used.

Comparing the results of the ammonification tests as a whole, using the casein and the dried blood methods, it is apparent that the latter allows of much greater differentiation between the ammonifying power of soils differently treated. The casein method permits of the securing of much better agreement among duplicate determinations, but this point is of minor importance to the securing of results distinguishing more widely between ammonification in different soils. Some further modification in

the technique of the casein method may remedy the difficulty mentioned but until such a change is made, the dried blood method must be considered the most satisfactory.

THE NITRIFICATION EXPERIMENTS

To determine the effect of the various materials used in this work on the nitrifying power of the soil, samples secured on the dates previously mentioned were tested by the ammonium-sulfate-fresh-soil method as has been described. The tests on February 12 were incubated for 27 days and all the other tests were made in 28 days. The results of the determinations are given in table VII, and the average results appear in table VIII. A few of the results are omitted from the averages because of evident abnormality. It will be noted, however, that as a whole the duplicate determinations agreed very satisfactorily.

From table VIII it appears that the differences in nitrifying power were not pronounced. In general, however, the cowpea hay and clover hay had the greatest action on the nitrifiers and the manures a smaller effect, while the straws, stover, and timothy hay showed little influence on nitrification. The differences were too small to warrant definite conclusions in the case of the three latter materials, and hence the only statement which can be made is that these materials exerted practically no influence on nitrification. The small variations in the nitrifying power of the soils used in this work might have been increased by a longer incubation period. Possibly larger differences might have been found with variations in the method employed, but from the standpoint of these experiments it is apparent that the nitrogen-carbon ratio of the materials used had no effect on the influence of the substances on the nitrifying power of the soils. The effects of the materials were dependent probably, as in the case of ammonification, on the chemical compounds present in the materials.

It is interesting to note that the manure, which exerted the greatest effect on ammonification, showed also comparatively large effects on nitrification, while the legume hays, which showed smaller effects on ammonification than the manures, gave a greater influence on nitrification. Just why this should be is difficult to determine, as ordinarily materials which favor ammonification in field soils will favor also nitrification, unless the amounts of organic matter added are so large that nitrification is entirely inhibited. It seems to be a matter of considerable doubt at present whether it is possible to add sufficient organic matter to field soils to prevent nitrification. However that may be, it is apparent here that nitrification was not restricted by any of the maximum applications of the common materials used and ammonification was increased as described, hence it might be ex-

TABLE VII. THE NITRIFICATION OF AMMONIUM SULFATE

Pot No.	Lab. No.	I December 24		II January 7		III January 28		IV February 12		V February 26		VI March 12	
		Nitrate Mgs. N.	Average Mgs. N.	Nitrate Mgs. N.	Average Mgs. N.	Nitrate Mgs. N.	Average Mgs. N.	Nitrate Mgs. N.	Average Mgs. N.	Nitrate Mgs. N.	Average Mgs. N.	Nitrate Mgs. N.	Average Mgs. N.
1	1	20.41		19.74		17.04		20.83		22.06		24.19	
	2	20.83	20.62	19.99	19.36	17.04	17.04	21.43	21.13	21.74	21.90	23.40	23.79
2	3	20.83		18.99		17.04		19.74		20.27		24.57	
	4	20.83	20.83	19.74	19.36	16.85	16.94	21.43	20.58	22.06	21.16	23.40	23.98
3	5	20.83		20.00		18.99		18.75		22.06		28.47	
	6	21.23	21.53	19.74	19.87	18.99	18.99	19.59	19.17	21.74	21.90	22.40	25.43
4	7	21.23		20.00		18.52		20.00		22.06		22.40	
	8	21.23	21.48	19.59	19.87	16.85	17.68	19.74	19.87	22.06	22.06	25.86	24.13
5	9	21.23		22.06		16.48		22.39		22.06		25.86	
	10	21.71	22.46	22.40	22.23	16.48	16.48	21.43	21.91	22.39	22.22	27.77	26.81
6	11	20.41		23.81		18.52		19.23		22.73		25.42	
	12	21.73	21.07	24.19	21.00	16.48	17.50	21.74	20.43	21.43	22.08	27.77	26.59
7	13	21.73		22.73		15.96		22.73		22.40		26.31	
	14	21.73	21.73	22.39	22.56	16.85	16.90	23.40	23.06	24.57	23.48	23.40	24.85
8	15	21.23		21.74		16.85		20.27		21.74		27.77	
	16	22.72	22.47	22.46	22.07	17.04	16.94	20.27	20.27	24.57	23.15	24.19	25.98
9	17	20.83		21.13		17.04		20.55		22.73		23.81	
	18	21.23	21.03	20.83	20.98	16.85	16.94	20.55	20.55	19.74	21.23	22.40	23.10
10	19	21.23		21.13		17.04		18.07*		22.06		26.31	
	20	20.83	21.03	21.13	21.13	16.85	16.94	17.65*	.....	18.75*	22.06	23.81	25.06
11	21	20.83		20.27		17.65		18.99		22.06		25.42	
	22	21.23	21.03	21.45	20.37	16.99	17.32	20.83	19.91	20.27	21.16	26.31	25.86
12	23	21.23		18.75		18.07		20.27		22.06		25.42	
	24	20.83	21.03	18.99	18.87	15.96	17.01	22.06	21.16	19.23*	22.06	22.40	23.91
13	25	20.41		22.06		16.85		18.07*		20.00		25.42	
	26	20.83	20.62	21.74	21.90	17.04	16.94	20.27	20.27	20.27	20.13	23.81	24.61
14	27	20.83		22.06		16.66		19.74*		22.06		25.00	
	28	20.83	20.83	21.74	21.90	17.44	17.05	21.43	21.43	21.74	21.90	24.57	24.78
15	29	21.23		21.74		18.99		22.73		22.73		27.27	
	30	21.73	21.48	24.59	25.61	18.75	18.87	22.40	22.56	23.81	23.27	25.42	26.34
16	31	21.23		25.86		19.48		22.40		24.19		27.27	
	32	21.23	21.23	25.42	25.64	18.52	19.00	23.81	23.10	23.40	23.79	25.86	26.56
17	33	21.23		23.81		18.52		20.55		22.39		27.77	
	34	21.73	21.48	23.40	23.60	17.04	17.78	22.73	21.64	20.55	21.47	25.42	26.59
18	35	21.23		23.40		18.52		23.40		24.19		26.78	
	36	22.22	21.72	23.40	23.40	17.65	18.08	20.00	21.70	20.83	22.51	27.77	27.27

* Results omitted from the averages.

pected that the effects would be in the same direction for both processes. It is possible, however, that different materials might increase both processes, but to different degrees.

It is important to note, however, from these results that the common humus-forming materials, such as are used on the farm, when applied in maximum amounts did not depress the nitrifying power of the soil, at least of this particular soil. On the other hand, there was an increase in nitrification to a more or less pronounced extent with the different materials. In the case of soils containing more organic matter, or material of a nar-

TABLE VIII. THE NITRIFICATION OF AMMONIUM SULFATE

Pot No.	Treatment	I		II		III	
		Nitrate N. Mgs.	Average Mgs. N.	Nitrate N. Mgs.	Average Mgs. N.	Nitrate N. Mgs.	Average N. Mgs.
1	Check	20.62		19.36		17.04	
2	Check	20.83	20.72	19.36	19.36	16.94	16.99
3	Horse manure	21.53		19.87		18.99	
4	Horse manure	21.48	21.50	19.78	19.82	17.68	18.33
5	Cow manure	22.46		22.23		16.48	
6	Cow manure	21.07	21.76	24.60	23.11	17.50	16.99
7	Rotted manure	21.73		22.56		16.90	
8	Rotted manure	22.47	22.10	22.07	22.31	16.94	16.92
9	Oats straw	21.03		20.98		16.94	
10	Oats straw	21.03	21.03	21.13	21.05	16.94	16.94
11	Corn stover	21.03		20.37		17.32	
12	Corn stover	21.03	21.03	18.87	19.62	17.01	17.16
13	Timothy hay	20.62		21.90		16.94	
14	Timothy hay	20.83	20.72	21.90	21.90	17.05	16.99
15	Cowpea hay	21.48		25.61		18.87	
16	Cowpea hay	21.23	21.35	25.64	25.62	19.00	18.93
17	Clover hay	21.48		23.60		17.78	
18	Clover hay	21.72	21.60	23.40	23.50	18.08	17.93

Pot No.	Treatment	IV		V		VI	
		Nitrate N. Mgs.	Average Mgs. N.	Nitrate N. Mgs.	Average Mgs. N.	Nitrate N. Mgs.	Average N. Mgs.
1	Check	21.13		21.90		23.79	
2	Check	20.58	20.85	21.16	21.53	23.98	23.88
3	Horse manure	19.17		21.90		25.43	
4	Horse manure	19.87	19.52	22.06	21.98	24.13	24.78
5	Cow manure	21.91		22.22		26.81	
6	Cow manure	20.43	21.17	22.08	22.15	26.59	26.70
7	Rotted manure	23.06		23.48		24.85	
8	Rotted manure	20.27	21.66	23.15	23.31	25.98	25.91
9	Oats straw	20.55		21.23		23.10	
10	Oats straw	.....	20.55	22.06	21.64	25.06	24.08
11	Corn stover	19.91		21.18		25.86	
12	Corn stover	21.16	20.53	22.06	21.61	23.91	24.88
13	Timothy hay	20.27		20.13		24.61	
14	Timothy hay	21.43	20.85	21.90	21.01	24.78	24.69
15	Cowpea hay	22.56		23.27		26.34	
16	Cowpea hay	23.10	22.83	23.79	23.53	26.56	26.45
17	Clover hay	21.64		21.47		26.59	
18	Clover hay	21.70	21.67	22.51	21.99	27.27	26.93

rower nitrogen-carbon ratio, it is difficult to predict the effect, but inasmuch as organic matter in such large amounts as were used here, particularly in the case of the leguminous green manures, would not be used unless the soils were low in nitrogen, it seems safe to say that there is no danger of restricting nitrification in soils by additions of amounts of organic matter such as would be used in the field.

In general from these experiments it is apparent that nitrification was increased by additions of organic materials, such as are made on the farm, and these increases were independent of the nitrogen-carbon ratio in the materials, although there were some indications that the materials of a narrower ratio gave a greater effect than those of a wider ratio, but the results were not conclusive. Inasmuch as the latter possibility is the opposite of the case with ammonification, it is apparent that more definite results must be secured before any conclusions should be drawn.

THE AZOFICATION EXPERIMENTS

The samples drawn on the six dates mentioned previously were tested for their azofying or nitrogen-fixing power by the fresh-soil method. At the first sampling, mannite (5 gms. per 100 grams of soil) was used and at the later dates dextrose was employed, being added from solution at the rate of 3 grams per 100 grams of soil.

The incubation period was 11 days, except in the case of the second sampling when the tests were allowed to incubate 14 days. The complete results of the tests are given in table IX and the summarized results appear in table X.

As might be expected, there were considerable variations in the results of the duplicate determinations. The method used for the determination of total nitrogen does not permit of the estimation of such small amounts of nitrogen as sometimes represent the nitrogen fixation. In some instances a smaller amount of nitrogen was actually found after the incubation period but it was hardly possible for any loss of nitrogen to occur and hence such results should be attributed to the fact that the method is not accurate for small amounts of nitrogen. These low results are eliminated from the averages and are interpreted merely as representing, therefore, the absence of any azofication.

In calculating the results, the total nitrogen present in the soils in the tests before incubation was estimated and subtracted from the nitrogen present at the end to determine the amount of nitrogen fixed. A slight error is, of course, introduced here in case not all of the mannite or dextrose added was used by the bacteria. Then the unused portion would be included in the sample analyzed after incubation. In such a case the results would be slightly lower than they should be, hence the amounts

TABLE IX. AZOFICATION

Pot No.	Lab. No.	N. in Orig. Soil Mgs.	I December 24			II January 7			III January 28			IV February 12			V February 26			VI March 12		
			N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.	N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.	N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.	N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.	N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.	N. after incub. Mgs.	N. fixed Mgs.	Average Mgs.
1	1	98.80	99.50	.70		110.10	11.30		102.03	3.50		102.30	3.50		103.70	4.90		106.50	7.70	
	2	98.80	98.80	.00	.35	110.20	1.40	6.35	111.50	12.70	8.10	100.90	2.10	2.80	100.90	2.10	3.50	105.10	6.30	7.00
2	3	98.80	102.30	3.50		104.40	5.60		105.80	7.00		98.80	.00		103.70	4.90		105.80	7.00	
	4	98.80	100.90	2.10	2.80	105.80	7.00	6.30	104.40	5.60	6.30	107.20	8.40	4.20	103.00	4.20	4.55	107.20	8.40	7.70
3	5	106.56	110.80	4.24		115.70	9.14		122.10	15.54		110.80	4.24		108.60	2.04		117.10	10.54	
	6	106.56	107.20	.64	2.44	115.70	9.14	9.14	116.40	9.84	12.69	107.90	1.34	2.79	110.10	3.54	2.79	118.50	11.94	11.24
	7	106.56	107.20	.64		115.00	8.44		116.40	9.84		112.20	5.64		107.20	.66		115.00	8.44	
4	7	106.56	107.20	.64		115.00	8.44		116.40	9.84		109.40	2.84	4.24	115.70	9.14	4.70	118.50	11.94	10.19
	8	106.56	105.10	−1.46*	.64	115.70	9.14	8.79	115.70	9.14	9.49	109.40	2.84		111.50	3.88		118.50	10.88	
5	9	107.62	110.10	2.48		119.90	12.28		121.30	13.68		111.50	3.88		119.90	12.28	8.08	125.60	17.98	14.43
	10	107.62	111.50	3.88	3.18	115.00	7.38	9.83	117.10	9.48	11.58	117.80	10.18	7.03	119.90	12.28		120.60	12.98	
6	11	107.62	110.10	2.48		118.50	10.88		108.20	−.42*		115.00	7.38		112.90	5.28	8.78	115.70	8.08	10.53
	12	107.62	111.50	3.88	3.18	119.20	11.58	9.48	119.20	11.58	11.58	111.50	3.88	5.63	115.00	3.68		115.00	3.68	
7	13	111.32	111.50	.18		117.80	6.48		117.10	5.78		115.70	4.38		115.00	3.68	4.03	127.70	16.38	10.03
	14	111.32	111.50	.18	.18	122.80	11.48	8.98	120.60	9.28	7.53	112.90	1.58	2.98	115.70	4.38		122.80	11.48	
8	15	111.32	105.80	−5.52*		119.90	8.58		122.80	11.48		121.30	9.08	7.18	113.60	2.28	4.73	127.00	15.68	13.58
	16	111.32	113.60	2.28	2.28	121.40	10.08	9.33	131.20	19.88	10.68	115.70	4.38		118.50	7.18		106.50	5.53	
9	17	100.97	104.40	3.43		105.80	4.83		112.20	11.23		115.70	14.73		108.60	7.63	7.63	108.60	7.63	6.58
	18	100.97	107.20	6.23	3.83	104.40	3.43	4.13	112.90	11.93	11.58	111.50	10.53	12.63	108.60	7.63		104.40	3.43	
10	19	100.97	100.20	−.77*		112.90	11.93		117.10	16.13		104.40	3.43		105.10	4.23		112.90	11.93	7.66
	20	100.97	101.50	.53	.53	108.60	7.63	9.78	112.90	11.93	14.03	107.20	6.23	4.83	111.50	10.53	7.38	111.50	8.27	
11	21	103.23	105.80	2.57		109.40	6.17		107.20	3.97		108.60	5.37		107.20	3.97		106.50	3.27	5.77
	22	103.23	107.90	4.67	3.62	111.50	8.27	7.22	105.80	2.57	3.27	104.40	1.17	3.27	110.10	6.87	5.42	109.40	6.17	
12	23	103.23	100.90	2.33		108.60	5.37		111.50	8.27		112.90	9.67		107.90	4.67		110.10	6.87	6.52
	24	103.23	106.50	3.27	2.80	111.50	8.27	6.82	109.40	6.17	7.22	107.90	4.67	7.17	110.80	7.57	6.12	109.40	6.17	
13	25	100.75	103.00	2.25		105.80	5.05		105.80	5.05		104.40	3.65		107.20	6.45		105.80	5.05	
	26	100.75	105.10	4.35	3.30	114.30	13.55	9.30	104.40	3.65	4.35	108.60	7.85	5.75	104.40	3.65	5.05	112.90	12.15	8.60
14	27	100.75	102.30	1.55		108.60	7.85		103.00	2.25		106.50	5.75		107.20	6.45		110.10	9.35	
	28	100.75	107.20	6.45	4.00	109.40	8.65	8.25	108.60	7.85	5.05	104.40	3.65	4.70	112.90	12.15	9.30	109.40	8.65	9.00
15	29	107.55	108.60	1.05		112.20	4.65		112.90	5.35		110.10	2.55		118.60	6.05		108.60	1.05	
	30	107.55	107.20	−.35*	1.05	112.90	5.35	5.00	115.00	7.45	6.40	105.10	−2.45*	2.55	107.20	−.35*	6.05	110.10	2.55	1.80
16	31	107.55	107.20	−.35*		111.50	3.95		116.40	8.85		112.90	5.35		110.10	2.55		108.60	1.05	
	32	107.55	107.20	−.35*	.....	112.20	4.65	4.30	112.20	4.65	6.75	112.20	4.65	5.00	107.90	.35	1.45	112.20	4.65	2.85
17	33	107.03	110.10	3.07		111.50	4.47		113.60	6.57		110.80	3.77		110.10	3.07		109.40	2.37	
	34	107.03	103.00	−4.03*	3.07	108.60	1.57	3.02	114.30	7.27	6.92	108.60	1.57	2.67	111.50	4.47	3.77	110.10	3.07	2.72
18	35	107.03	108.00	.97		112.90	5.87		108.60	1.57		112.90	5.87		110.10	3.07		112.20	5.17	
	36	107.03	107.20	.17	.57	115.70	4.47	5.17	111.50	4.47	3.02	110.10	3.07	4.47	111.50	4.47	3.77	109.40	2.37	3.77

* Results omitted from the averages.

of nitrogen fixed from the atmosphere may be too low but that fact need not interfere with the interpretation of the results.

Considering the results given in table X, it is apparent that the addition of various organic materials to the soil influenced to a considerable extent the fixation of nitrogen by non-symbiotic bacteria. In some cases the amount of nitrogen fixed in the 11-day incubation period amounted to one-sixth of the nitrogen originally present in the soil.

The soils receiving cow manure and oats straw showed for the most part the greatest increase in azofying power and the influ-

TABLE X. AZOFICATION

Pot No.	Treatment	I		II		III	
		N. fixed Mgs.	Average Mgs.	N. fixed Mgs.	Average Mgs.	N. fixed Mgs.	Average Mgs.
1	Check	.35		6.35		8.10	
2	Check	2.80	1.57	6.30	6.32	6.30	7.20
3	Horse manure	2.44		9.14		12.69	
4	Horse manure	.64	1.54	8.79	8.96	9.49	11.09
5	Cow manure	3.18		9.83		11.58	
6	Cow manure	3.18	3.18	9.48	9.65	11.58	11.58
7	Rotted manure	.18		8.98		7.53	
8	Rotted manure	2.28	1.23	9.33	9.15	10.68	9.10
9	Oats straw	3.83		4.13		11.58	
10	Oats straw	.53	2.18	9.78	6.95	14.03	12.80
11	Corn stover	3.62		7.22		3.27	
12	Corn stover	2.80	3.21	6.82	7.02	7.22	5.24
13	Timothy hay	3.30		9.30		4.35	
14	Timothy hay	4.00	3.85	8.25	8.77	5.05	4.70
15	Cowpea hay	1.05		5.00		6.40	
16	Cowpea hay	....	1.05	4.30	4.65	6.75	6.57
17	Clover hay	3.07		3.02		6.92	
18	Clover hay	.57	1.82	5.17	4.09	3.02	4.97

Pot No.	Treatment	IV		V		VI	
		N. fixed Mgs.	Average Mgs.	N. fixed Mgs.	Average Mgs.	N. fixed Mgs.	Average Mgs.
1	Check	2.80		3.50		7.00	
2	Check	4.20	3.50	4.55	4.02	7.70	7.35
3	Horse manure	2.79		2.79		11.24	
4	Horse manure	4.24	3.51	4.70	3.74	10.19	10.71
5	Cow manure	7.03		8.08		14.43	
6	Cow manure	5.63	6.33	8.78	8.43	10.53	12.48
7	Rotted manure	2.98		4.03		10.03	
8	Rotted manure	7.18	5.08	4.73	4.38	13.58	11.80
9	Oats straw	12.63		7.63		6.58	
10	Oats straw	4.83	8.73	7.38	7.50	7.66	7.12
11	Corn stover	3.27		5.42		5.77	
12	Corn stover	7.17	5.19	6.12	5.79	6.52	6.14
13	Timothy hay	5.75		5.05		8.60	
14	Timothy hay	4.70	5.22	9.30	7.17	9.00	8.80
15	Cowpea hay	2.55		6.05		1.80	
16	Cowpea hay	5.00	3.77	1.45	3.75	2.85	2.32
17	Clover hay	2.67		3.77		2.72	
18	Clover hay	4.47	3.57	3.77	3.77	3.77	3.24

ence of the rotted manure was only slightly less than that of the cow manure.

The horse manure gave less effect than the other manures and about the same in most cases as the timothy hay. The corn stover also affected the azofying power of the soil to about the same extent as the horse manure.

The cowpea hay and the clover hay exerted the smallest effect of any of the materials on the azofying power of the soil.

It appears, therefore, from the results as a whole that the nitrogen-carbon ratio of the various humus-forming materials applied to the soil was of little significance from the standpoint of the effect on azofication. The influence of the materials was exerted regardless of the nitrogen-carbon ratio. Thus the oats straw of a wide ratio and the cow manure of a narrower ratio had about the same effect. Similarly the timothy hay and the horse manure of wide and narrow ratios respectively had considerable influence. Again the rotted manure of a very narrow ratio exercised as much effect on azofication as the timothy hay which had a wide ratio.

To just what influence of the materials the difference in results was due is difficult to determine. It may be that the difference in chemical composition of the substances explains the results. This was the conclusion reached in the ammonification and nitrification experiments and would probably hold true here. It is well known that organic compounds of different composition exert quite different effects on the azotobacter and hence the results from the use of the materials employed here might be expected, to the extent at least that the different materials had various effects. The important point in this connection which these results bring out is that the character of the compounds present apparently determined the results and the ratio of the nitrogen to carbon present gave no indication of the effects to be expected.

It is interesting to note further that the leguminous hays had much less effect on the azofying power of the soil than the other materials. Especially is this point worthy of mention because of the relative effects of the legumes and non-legumes as green manures. If the latter materials will increase the fixation of nitrogen from the atmosphere by the non-symbiotic azotobacter sufficiently to supply as much nitrogen for the use of crops as is added in legume crops, such materials might frequently be preferable for use on soils. It is impossible from these results to ascertain whether such is the case or not. Further results must be secured with complete field experiments before definite conclusions can be reached.

These results do show, however, that the non-legumes increased the azofying power of the soil to a much greater extent than the

legumes. This greater effect was probably due as has been mentioned to the chemical composition of the materials. In this case the effects are in the same direction as the widening of the nitrogen-carbon ratio, and it might seem that the ratio of the materials would indicate the influence on azofication, but inasmuch as the manures of narrower nitrogen-carbon ratio had as much effect as the non-legumes and straws it would evidently not be warranted to draw any conclusion regarding the effects of the ratio in materials added on azofication.

In general, then, the results show that azofication was favored by manure to a large extent; that straw, stover, and non-leguminous hays had almost as great an effect as the manures, altho of a much wider nitrogen-carbon ratio, and that the leguminous hays had the least effect of any of the materials used in the experiment. Apparently the nitrogen-carbon ratio of the materials used was of little or no significance in indicating their influence on azofication and differences in effects were due rather to variations in the chemical compounds present.

There are indications, however, that non-leguminous hays and straws may increase azofication in soils to a large enough extent to make their use more profitable than that of legumes which altho adding nitrogen to the soil are somewhat more expensive to use.

These conclusions apply, of course, as must be emphasized again, only to this particular soil type and when the materials are used in amounts such as are employed here, that is, in maximum field applications. The results are, therefore, directly applicable to farm conditions on this soil type and may indicate what will occur on similar soils. Further experiments on the comparative effects of legumes and non-legumes as green manures from the standpoint of their influence on azofication are extremely desirable and may lead to important practical conclusions.

One point further is worthy of mention in connection with these experiments and that is that the results secured with dextrose were much more satisfactory than those with mannite. The latter material has been considered the best for such work, but it is possible that the cheaper dextrose may serve as well or even better. The point is worthy of consideration in connection with extensive azofication experiments.

Comparing the azofication results as a whole with the ammonification and nitrification results, it appears that there was little similarity in the effects of the different materials on the different processes. Azofication was increased in some cases to a greater extent by some materials than by others, whereas the opposite was the case with ammonification and nitrification.

This fact brings up another important point in connection

with the use on soils of organic materials which increase azofication to the greatest extent. Is it necessary that ammonification and nitrification should also be considered? This is a question which must be left for future rather extensive experiments to settle, and involves the whole question of the form in which plants may assimilate their nitrogen, a question which is apparently far from definitely settled as yet.

THE CROP YIELDS

The crop of oats on the pots, the duplicates of which were kept bare for bacteriological tests, was harvested just prior to maturity, dried, ground, and analyzed for total nitrogen. The green and dry weights of the crop are given in table XI and the nitrogen content of the crop together with the calculated removal of nitrogen from the soil are given in table XII.

TABLE XI. THE CROP YIELDS

Pot No.	Treatment	Green Weight Gms.	Average Gms.	Dry Weight Gms.	Average Gms.
19	Check	189.0		58.6	
20	Check	207.0	198.00	61.5	60.05
21	Horse manure..	112.9		30.3	
22	Horse manure..	119.7	116.30	32.0	31.15
23	Cow manure....	234.4		70.6	
24	Cow manure....	218.2	226.30	63.0	66.80
25	Rotted manure.	279.6		85.2	
26	Rotted manure.	298.4	289.00	88.9	87.05
27	Oats straw.....	115.3		32.5	
28	Oats straw.....	100.6	107.95	28.9	30.70
29	Corn stover....	182.9		50.0	
30	Corn stover....	180.7	181.80	52.6	51.30
31	Timothy hay...	143.2		44.6	
32	Timothy hay...	151.1	147.15	45.3	44.95
33	Cowpea hay.....	360.5		72.4	
34	Cowpea hay.....	244.6	252.55	72.8	72.60
35	Clover hay.....	224.9		63.6	
36	Clover hay.....	224.9	224.90	64.0	63.80

Examining the yields in table XI, it appears that the rotted manure, the cow manure, and the leguminous hays increased the crop yields to a considerable extent. The horse manure depressed the yield over that of the untreated soil. The plants in these pots were weak and turned yellow soon after they appeared above the surface of the soil, but after about ten weeks this bad effect from the horse manure disappeared and the oats showed a more vigorous growth. If the experiment had continued longer, it is probable that the yields would have equaled those secured with the other materials. The depressing action was probably due to the introduction with the heavy application of manure of chemical substances which were injurious to the young plants.

All of the non-legume hays, straw, and stover materials with a wide nitrogen-carbon ratio gave no gain in the crop yields. In fact, an actual depression in yields occurred. These materials

apparently did not decompose sufficiently rapidly to aid the crop grown or the nitrogen content of the soil was more depleted than was believed. At any rate, the legume hays increased the yields, which would indicate that the nitrogen factor on these soils was important and that the non-legumes did not increase the fixation of nitrogen from the atmosphere sufficiently to keep the oats supplied with that element.

The experiment, of course, was hardly continued long enough for definite crop results to be secured and a second crop was planted after the first was removed in order to determine whether different results would be secured, allowing a longer time for the organic material to decompose.

It appears from these first results, however, that the nitrogen-carbon ratio of the organic materials was of considerable significance in determining the effects of the materials used on the crop yields from this particular soil. In every case those substances with the narrower nitrogen-carbon ratios increased to the greatest extent the crop yields, while the materials of wide ratios decreased the crop yields. The nitrogen factor was evidently very important on this particular soil.

In table XII, it is seen that the percentage of nitrogen in the oats varied considerably, the tendency being for the lowest yields to show the highest nitrogen content. The largest crops, however, removed the greatest amount of nitrogen from the soil.

The crop yields as a whole show that materials such as were used in this work may exert a considerable influence on bacterial activities and not show the same effect on the crop grown. The

TABLE XII. THE ANALYSES OF THE CROPS

Pot No.	Treatment	N. in Crop Per cent	Average Per cent	C. in Crop Per cent	Average Per cent	N—C Ratio	N. removed Gms.	Average Gms.
19	Check	.734		39.429			.430	
20	Check	.730	.732	37.637	38.533	1 : 52.64	.449	.439
21	Horse manure	.818		41.911			.248	
22	Horse manure	.861	.839	37.083	39.497	1 : 47.07	.276	.262
23	Cow manure	.734		45.827			.518	
24	Cow manure	.734	.734	45.372	45.599	1 : 62.11	.462	.490
25	Rotted manure	.776		40.413			.661	
26	Rotted manure	.797	.786	37.367	38.890	1 : 49.48	.709	.685
27	Oats straw	.764		38.745			.248	
28	Oats straw	.771	.767	39.140	38.943	1 : 50.77	.224	.236
29	Corn stover	.783		39.195			.392	
30	Corn stover	.797	.790	37.254	38.225	1 : 48.37	.419	.405
31	Timothy hay	.709		38.653			.316	
32	Timothy hay	.703	.706	39.985	39.319	1 : 55.69	.318	.317
33	Cowpea hay	.903		38.372			.654	
34	Cowpea hay	.868	.885	38.808	38.590	1 : 43.60	.632	.643
35	Clover hay	.805		42.410			.512	
36	Clover hay	.836	.820	44.443	43.427	1 : 52.96	.534	.523

effects on subsequent crops, however, would be a more definite indication of the relative values of these materials, because of the need of time for decomposition. In other words, it would not be expected that the effects of such materials on crops would be exerted as soon as effects on bacterial activities. The latter must always precede the former. Hence some time should elapse after applying organic materials before the effect on the crop grown is determined. If the effects of materials of wide nitrogen-carbon ratio are dependent to any extent on the increase in nitrogen content of the soil through non-symbiotic nitrogen-fixation, time should be allowed for this process to occur before the comparative effects on crop yields are tested. It is not regarded, therefore, that these crop yields present facts which oppose in any way the possibility of sufficient azofication occurring in soils treated with non-legumes to equal the effects caused by legumes.

THE SECOND CROP YIELDS

The second crop of oats grown on the same soils as in the case of the first crop was harvested before it had attained any considerable growth. The yields given in table XIII, however, show some interesting relations to those of the first crop.

In this case, all the treatments increased the oats growth but the horse and cow manures gave the largest effect here, while the rotted manure gave a smaller effect than any of the other materials. With the first crop, the rotted manure gave the greatest influence, while the cow manure hardly increased the yield and the horse manure depressed the oats growth. Evidently the cause of the injurious action of the horse manure had disappeared before the second crop was grown, and only beneficial effects from the material were in evidence on the second crop.

TABLE XIII. THE SECOND CROP YIELDS

Pot No.	Treatment	Green Weight Gms.	Average Gms.	Dry Weight Gms.	Average Gms.
19	Check	26.7		6.5	
20	Check	32.3	29.50	7.7	7.1
21	Horse manure	47.7		11.6	
22	Horse manure	57.0	52.35	12.7	12.15
23	Cow manure	49.9		12.8	
24	Cow manure	57.95	53.92	15.0	13.9
25	Rotted manure	34.0		7.4	
26	Rotted manure	39.5	36.75	9.2	8.3
27	Oats straw	41.7		10.0	
28	Oats straw	49.55	45.62	12.2	11.1
29	Corn stover	56.65		11.5	
30	Corn stover	43.2	49.92	10.0	10.75
31	Timothy hay	38.6		7.0	
32	Timothy hay	43.4	41.00	11.2	9.1
33	Cowpea hay	37.3		9.0	
34	Cowpea hay	51.1	44.20	12.2	10.6
35	Clover hay	44.5		9.5	
36	Clover hay	41.15	42.82	9.5	9.5

The rotted manure had apparently lost much of its value for increasing the crop yield by the time the first crop was removed, and had little effect on the second crop.

The oats straw and corn stover gave greater yield than the legume hays and the timothy hay had only a slightly smaller effect than the clover and cowpeas. It is apparent, therefore, that the conclusion drawn in connection with the first yields was well warranted. The non-legumes here seemed to have a greater or as great an effect on the crop as the legumes. Evidently the nitrogen fixed by azofiers was sufficient to supply the second crop of oats with as much of that element as was furnished by the legumes. Of course, there was probably some neutralizing action here as might be expected. If the first crop of oats took out much more nitrogen where the legumes were used than where the other materials were applied, the second crop might be not as well supplied as in the case of the non-legumes because of a shortage of nitrogen. Such could hardly be the case here, however, to more than a negligible extent and hence the conclusion seems justified that non-legumes may be as beneficial as legumes on crops grown, provided sufficient time is allowed to elapse between the application of the materials and the growth of the crop, for decomposition to occur and the fixation of nitrogen from the atmosphere to take place.

There is much closer agreement between the effects of the various materials on bacterial activities and on the second crop of oats than with the first crop. It will be recalled that the first crop of oats was seeded as soon as the substances were added, and it would appear from these results that the influence of many of these common humus-forming substances on crops is much greater if time is allowed for decomposition and other affected bacterial processes to occur before the crop is grown.

The nitrogen-carbon ratio of the various substances did not seem to be of as much importance in determining their effect on the second crop of oats as with the first crop, although there were indications that the materials with wider ratios had more effect than those with narrower ratios.

SUMMARY

The results of these experiments on this particular soil type lead to the following conclusions.

1. Applications of the common humus-forming materials in maximum amounts for farm conditions and in a dried condition increased bacterial activities, ammonification, nitrification, and azofication to a considerable extent.

2. The manures, horse manure, cow manure, and rotted manure gave the greatest effect on ammonification in most cases, altho timothy hay surpassed the horse manure and cow manure

in the extent of its effect in several instances. The oats straw and corn stover gave a smaller effect than the manures and the legume hays, clover, and cowpeas showed the least effect on ammonification of any of the materials used.

3. Increases in ammonification due to the applications of humus-forming materials were independent of the nitrogen-carbon ratio of the materials added and were probably dependent on the chemical composition of the substances.

4. The relative effects of the various materials used would undoubtedly be somewhat altered for field conditions, because of the fact that they were applied in a dried condition. Especially in the case of the manures would the influence on ammonification be accentuated because of the actual addition of bacteria to the soil.

5. The dried-blood-fresh-soil method gave better results for ammonification than the casein-fresh-soil method. The latter gave better duplicate results, but the differences between different soils were not nearly so pronounced. Some further modification of the casein method seems necessary for its general use.

6. Nitrification was increased in much the same way as ammonification, by the various organic materials. The leguminous green manures exerted, however, somewhat greater effects than the manures, and also more influence than the non-legumes. These results were the opposite of those secured with ammonification, but the differences were not great enough to permit of definite conclusions.

7. Increases in nitrification brought about by the various materials were apparently independent of the nitrogen-carbon ratio in the substances. Indications of a greater effect of materials of a narrower ratio over those of a wide ratio cannot be considered conclusive.

8. Azofication or non-symbiotic nitrogen fixation was favored by manure to a large extent. Straw, stover, and non-leguminous hays had almost as great an effect as the manures and the leguminous hays had the least effect of any of the materials used.

9. The nitrogen-carbon ratio of the materials employed were of little or no significance in indicating their effects on azofication. There were indications, however, that non-legumes and straws might increase azofication in soils to a large enough extent to make their use more profitable than that of legumes which add nitrogen to the soil but are somewhat more expensive to use.

10. Further experiments carried on under field conditions to ascertain the relative effects of legumes and non-legumes on azofication are extremely desirable and results secured may be of great practical importance.

11. Dextrose gave better results in the azofication experi-

ments than mannite and may, therefore, be substituted for the more expensive material.

12. There was little similarity between the effects of the different organic materials on the different bacterial processes. Is it necessary that the material which increases ammonification, nitrification, and azofication be chosen for use in soils, or shall an increase in azofying power be sufficient to recommend the substance? This question cannot yet be answered.

13. The manures and legumes increased the first crop of oats, except in the case of the horse manure, which apparently exerted an injurious effect on the crop in its early stages of growth. This injury was disappearing and might have been unnoticed had the crop been grown for a longer period.

14. The substances with wide nitrogen-carbon ratio decreased the crop yield while those of narrow ratios gave increases. The nitrogen factor was evidently very important on this soil.

15. The nitrogen-carbon ratio of the organic materials did seem to be of importance in determining the influence on the first crop of oats.

16. If opportunity is to be given for non-legumes to exert as good an effect as legumes by increasing azofication to a sufficient extent to offset the nitrogen supplied by the legumes, the organic materials must be allowed sufficient time for considerable decomposition to occur before a crop is grown to test the effects.

17. The influence of the various substances applied to the soils was noted on a second crop of oats, but the relative effects were different. The non-legumes had as great an influence as the legumes and hence previous conclusions are confirmed that with the use of the former materials sufficient time must be allowed to elapse for azofication to occur if as beneficial effects are to be secured as with legumes.

18. The nitrogen-carbon ratio of the materials applied to the soil did not seem to be of as much importance in determining the effect on the second crop of oats as in the case of the first crop.

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The Relative Influence of Microorganisms and Plant Enzyms on Corn Silage Fermentation

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The Relative Influence of Microorganisms and Plant Enzyms on Corn Silage Fermentation

BY ALVIN R. LAMB

HISTORICAL

Ever since the fermentation of silage has been studied and discussed the question of the agent causing the fermentation has been in controversy. Some investigators have made the statement, based on evidence more or less incomplete, that microorganisms are solely responsible for the changes undergone by the ensiled forage. Other workers, who have based their conclusions on equally incomplete data, have held that in silage produced under proper conditions bacteria and yeasts do not figure to any appreciable extent, but that the plant cell itself is the cause of the chemical changes which take place in its constituents. Still other writers have sometimes taken sides on the subject without presenting any new data bearing on the problem.

Among the earliest workers on the chemistry and biology of silage formation were Burrill and Manns (3),[1] who found many species of bacteria in the silage, and stated that they were the cause of the chemical changes. Babcock and Russell (1) made silage in the presence of chloroform, ether, and benzene, obtaining in each case a change of color, some increase in acidity, and typical silage odor and flavor. These results and deductions made from other observations on the gases of the silo, the number of bacteria found, and on silage made from mature and immature corn led them to believe that bacteria were nonessential and that the cause of the fermentation was mainly the intramolecular changes which occur in protoplasm under anaerobic conditions when ordinary metabolic processes are suspended. Harding (8), working under their direction, found bacteria but no constant flora in silage. E. J. Russell (17) came to the conclusion that the primary and essential changes in silage fermentation were brought about by the plant cell and its enzyms and that the changes caused by bacteria were secondary and nonessential. Esten and Mason (6) found such large numbers of bacteria and yeasts in silage that they considered them the only important factor concerned. Hunter

[1] Reference is made by number to bibliography, pp. 336-331.

and Bushnell (10) recently found large numbers of the *Bacterium bulgaricus* group in silage and considered their activities very important. All the work mentioned above has been done with silage made from the corn plant (*Zea mays*), which is the chief silage crop in this country.

Investigators in plant physiology have found evidence of the evolution of carbon dioxid and the formation of alcohol under aseptic conditions in the tissues of many plants, including maize. The distinction as to whether this is due to the action of enzyms within the cells or to respiratory activities of the cell protoplasm is not made; but similar respiratory changes appear to be common to the majority of plants, especially in their seeds. It has been suggested that the respiration of plants under anaerobic conditions is identical with alcoholic fermentation. In many cases some of the alcohol is further oxidized or otherwise changed, but the ratio of carbon dioxid to alcohol is often found to be comparable to that of ordinary alcoholic fermentation by the zymase of yeast. Of course, anaerobic conditions obtain in the silo after the first few hours. Doroféjew (4) found that the respiration of injured leaves was accelerated. This may have some significance in connection with the chopping of corn before it is ensiled. Zaleski and Reinhard (27) and others have noticed similar effects in wounded vegetable tissues. With various seeds placed under anaerobic conditions Godlewski and Polzeniusz (7), Stoklasa et al. (20, 21), Minenkoff (13), and a number of others have found that alcohol and carbon dioxid are produced. Similar results have been obtained by others with other plant tissues than the seeds. Although Mazé and Perrier (12) have questioned the results of Stoklasa, it is still possible that such respiratory activities play a part in the formation of silage.

Certain enzyms have been shown to be present in corn grain. A proteoclastic enzym has been found by Vines (24) and likewise by Scheunert and Grimmer (18), who found an amylase also present. Sigmund (23) found a lipase in both the resting and germinating seeds of maize. Price (15) demonstrated the presence of a peroxidase, a catalase, a protease, an invertase, and a glucosidase in cornstalks. White (26) found proteoclastic and amyloclastic enzyms in maize seeds, which retained their activity in seeds 20 years old.

STATEMENT OF PROBLEM

A sharp differentiation between the activities of enzyms and microorganisms in a given medium is practically impossible. Both are susceptible to injury and destruction by heat and are more or less similarly subject to the inhibitive effect of our

common antiseptics. Moreover, some of the principal manifestations of bacterial activity are identical with those of enzym action, as, for example, the evolution of carbon dioxid, production of alcohol, rise in temperature of medium, and hydrolysis of protein. Antiseptics, if used in high enough concentration to inhibit all bacterial growth, seem also to exercise a deleterious effect on the plant tissues and their enzyms. Aseptic conditions can be maintained for respiration experiments on small amounts of plant tissue, but it would be very difficult to produce silage under such conditions. The problem attacked in the work at the Iowa Agricultural Experiment Station here reported was therefore to differentiate as accurately as possible between the results of the various activities of these two kinds of agents. A number of different experimental methods were employed in the effort to arrive at a distinction between them.

EXPERIMENTAL METHODS

Silage made in the laboratory in glass jars has been used in the greater part of this work. Experimental conditions can in this way be easily controlled, and comparisons are thus possible. The corn was chopped in a small silage cutter or was taken from the college farm silage cutter and packed as tightly as possible into cylindrical wide-mouth jars which were closed with rubber stoppers. Each was provided with an outlet tube for excess gases, which was closed with a pinchcock. Silage made in this manner is perfectly and normally preserved with characteristic appearance and aroma. Comparisons of chemical data between silage from the farm silos and from laboratory silos show no considerable difference. Of course, no two lots of silage are ever exactly alike chemically. The writer has previously made both corn silage and mixed silages in this manner (9, 11). Results obtained show no evidence that this laboratory silage is essentially different from silage made from similar material in an air-tight farm silo.

Some of the corn used in this work was grown to maturity in the greenhouse. Both greenhouse corn and field-grown corn were used, to prevent any possible abnormal results. The greenhouse corn was generally nearly as good in quality as the field-grown corn.

The analytical methods used are based upon the characteristic chemical changes which take place in silage fermentation. The formation of acids and alcohols, the evolution of carbon dioxid, the disappearance of simple sugars, and the degradation of protein, which are the principal chemical phenomena of the fermentation, have been measured by the methods described

below. Results from methods based on these chemical changes show, as the conventional estimations of crude protein, fiber, ether extract, etc., do not, the nature and extent of fermentation and the character of the silage, as nearly as chemical analysis can show. In each case comparisons were made with similar figures obtained on samples of the green corn from which the silage was made. In each case the chemical determinations were made upon the juice expressed from the silage in a Buchner press, under a pressure of 300 to 400 kgm. per square centimeter. This method of sampling facilitates the chemical examination, insures a well-mixed sample, and makes possible more comparable results.

The methods used are as follows:

Total acidity.—Ten c. c. of silage juice were diluted to about 500 c. c. with carbon dioxid free water, and titrated with decinormal barium hydroxid solution in the presence of phenolphthalein till a distinct pink appeared by reflected light against a white background.

Volatile acidity.—One hundred c. c. of juice were distilled in a current of carbon dioxid free steam. To hasten the liberation of volatile acids and alcohols, 100 gm. of sodium chlorid were added to the juice. About 600 c. c. of distillate were titrated with baryta water in the presence of phenolphthalein.

Alcohols (Distillation method).—The distillate from the volatile acid determination was neutralized with baryta water (solid phenolphthalein being added) and concentrated by repeated distillation with sodium chlorid (2). About 50 c. c. of alcohol solution were oxidized[1] in a pressure flask in a boiling water bath for 30 to 40 minutes, and the volatile acids then distilled off four or five times, with additions of carbon dioxid free water. The volatile acids were titrated and calculated as ethyl alcohol.

Alcohols (Aeration method).—In this method (5) a current of air was drawn thru the silage juice, which was saturated with ammonium sulphate, into concentrated sulphuric acid. The sulphuric acid-alcohol solution was then oxizided with the potassium dichromate solution, and distilled and calculated as in the previous method. Although other alchols are formed in silage in small amounts, all were calculated as ethyl alcohol.

Total sugars.—Fifty c. c. of juice were clarified with neutral lead acetate, the excess lead precipitated with anhydrous sodium carbonate, an aliquot allowed to stand 24 hours with hydrochloric acid, neutralized, and the total reducing sugars determined on an aliquot by either the Defren-O'Sullivan method or

[1]The oxidizing solution used was made up in the following proportions: 10 gm. of potassium dichromate ($K_2Cr_2O_7$), 20 gm. of sulphuric acid (H_2SO_4), 70 gm. of water.

the slightly modified volumetric method of Schoorl (19). In any given series the same method was used to insure comparable results.

Amino nitrogen.—The amino nitrogen was determined on the diluted juice with the Van Slyke apparatus (22). This determination shows the relative degree of hydrolysis of protein if used on the same or similar material at successive periods.

Ammonia nitrogen.—A 50-c. c. sample of the juice was distilled with magnesium oxid, according to the official fertilizer method.

Moisture.—Samples of about 100 gm. of silage were dried to constant weight, in most cases in a vacuum oven at 60° C.

SILAGE MADE UNDER ANTISEPTIC CONDITIONS

Corn silage made in the presence of ether and chloroform, as done by Babcock and Russell (1), is, of course, well preserved and evolves, after the antiseptic has been allowed to evaporate, an odor quite aromatic and characteristic of silage. The exact amount of antiseptic which should be added to inhibit bacterial growth without seriously impairing enzymic action is, however, very difficult to estimate. Moreover, Wagner (25) has found that certain bacteria may flourish in the presence of benzene, phloroglucinol, phenol, and phenolic derivatives. Some experimental silage was made, however, in the presence of chloroform, toluene, and cresol. Analytical data on these are shown in table I.

TABLE I.—ANALYTICAL DATA ON ANTISEPTIC SILAGE

(Calculated on basis of 100 gm. of dry silage.)

Kind of silage	Total acidity (N/10)	Alcohol	NH_2—N
	C. c.	*Gm.*	*Gm.*
Typical green corn	84	0	0.052
Toluene silage (2 per cent)	31	Trace.	.057
Chloroform silage (5 per cent)	250	.....	.176
Cresol silage (0.5 per cent)	255	0.15	.199
Normal silage	675	.81	.256

The amount of toluene added seems to have been enough to stop practically all change. No bacterial growth was obtained from the chloroform silage. It seems likely that the jar of cresol silage contained a limited number of active organisms, as two forms were isolated from this silage, one of them an acid former, and no evidence of spore formation could be obtained with either of these organisms. Some of the results of the work with antiseptics have been introduced here to show the apparent impossibility of obtaining conclusive results, at least with silage, by using such methods alone. These data, however, may be of

some value when compared to other data shown on the following pages. Further experiments with varying amounts of antiseptics were not attempted, as it seemed likely that other methods would give more conclusive results.

OTHER EXPERIMENTAL SILAGE

An effort was made to learn the effect of bacteria and yeasts alone, by heating jars of chopped corn to destroy the enzyms, followed by inoculation with an infusion of normal silage, which should carry the normal mixed flora of silage. After inoculation the jars were in each case incubated at 28° to 30° C., with a control jar of normal silage made from the same sample of corn. The analytical data, which are interesting but not conclusive, are shown in table II.

TABLE II—SILAGE HEATED AND INOCULATED

(Data on 100 c. c. of juice)

Kind of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2 N.
	C. c.	*C. c.*	*Gm.*	*Gm.*
Green corn	23	1.8	0	0.044
Normal silage (control)	375	118.0	.265	
Corn heated to 80° and inoculated	274	123.6	.345	.135
Green corn	43		0	.111
Normal silage	318	109.6	.150	.214
Corn heated to 90° and inoculated	198	99.1	.244	.068
Green corn	19.5	1.2	0	.024
Normal silage	342.5	73.1	.297	.114
Corn heated to 85° and inoculated	301.5	58.4	.188	.035

The prominent part which may be played by yeasts in the fermentation of silage under certain conditions was demonstrated by adding to a jar of silage sufficient tartaric acid in solution to make 2 per cent of the weight of the silage. An acid mixture of this strength practically inhibits bacterial action and favors the development of yeasts. A comparison of the acidity of the silage with the amount of tartaric acid added showed that evidently no other acid had been formed, except some volatile acid, which might possibly have come from the oxidation of alcohol. The quantity of alcohol found in 100 c. c. of this silage juice was 1.746 gm., expressed as ethyl alcohol, while normal silage juice contain only from 0.20 to 0.45 gm.

RATE OF CHEMICAL CHANGES IN SILAGE FERMENTATION

A more conclusive method of differentiating between the activities of enzyms and of microorganisms was suggested by a paper by Rahn (16), who discussed the usefulness of curves

in the interpretation of microbial and biochemical processes. It is shown by Rahn that the curve which is obtained when the formation of products of fermentation or other biochemical process is plotted, taking as abscissæ the total time elapsed and as ordinates the total amounts of compounds produced, is in many cases indicative of the nature or cause of the change. If the change is caused by enzymic action and is, therefore, purely chemical, the active mass of the agent causing the change does not increase as the reaction progresses; and the decreasing concentration of the substance acted upon and the accumulation of end products tend to decrease the rate of change. Thus, the curve becomes convex toward the Y axis. The mass of enzym does not increase unless there are living cells present to elaborate more enzym. However, if organisms are present and are active, they multiply until the exhaustion of nutrients or the accumulation of end products retards and finally stops their increase. Until this time the rate of change increases with the number of organisms, and the resulting curve is convex toward the X axis. Then there is a point of inflection, after which the curve becomes similar to the enzymic curve. An example of a curve of this type is shown in fig. 1, a typical "fermentation curve". There is always a point of inflection, or change in direction of curvature in a bacterial curve, provided the data begin before the number of organisms has reached its maximum. These two kinds of curves are discussed at some length by Rahn in the article cited (16).

In natural or mixed fermentations, such as the formation of silage, it is possible that different processes taking place at the same time will destroy the natural form of the curve. Or if both chemical and biological factors are present and producing the same substance, e. g., alcohol, the nature of the curve might be variable, depending upon the relative "active mass" of the two agents. In the case of so large an inoculation that the bacteria do not multiply materially, a bacterial curve might possibly resemble the curve of an enzymatic process. However, the chemical composition of the material and the extent of the fermentation depend upon many variable factors, such as soil and meteorological conditions, the method and rate of filling the silo, and opportunity for inoculation. The silage resulting is thus a variable product, depending on these and other factors. Therefore it seems very unlikely that any considerable error in the interpretation of curves drawn from silage data would persist thru a number of entirely separate experiments. This is evident from the data given in the following pages.

The analytical methods outlined above were used to obtain data on the chemical changes occurring during the early period

of silage fermentation, in order that curves such as just described might be plotted. The data obtained by Neidig (14) on this part of the fermentation process indicate the impossibility of obtaining regular curves and strictly comparable results by taking samples from a farm silo, owing to the variability in composition of the silage in different parts of the silo, and the necessity of the perfect exclusion of air thruout the process. Therefore, silage was made in the laboratory in small jars as mentioned above. The chopped corn was very thoroly mixed, and after a sample had been taken for the initial analysis, it was packed tightly into jars. The jars were kept under the same conditions, generally in an incubator at 28° to 32° C., following the average rise of temperature in the silo. One jar was opened each day and the juice pressed out and analyzed. This method should give as nearly correct results on the rate of change as it is possible to obtain, it being granted that silage formation in small jars is perfectly normal. The changes which were found were very similar to the changes observed by Neidig in the farm silos, except that the uniformity of the samples gave much more uniform and regular curves.

A series of nine jars of silage (series 1), using corn grown to maturity in the greenhouse, gave very interesting results. The corn was mixed and ensiled as described above. The determinations on the green corn and on each day's sample of silage juice were made, as usual, in a strictly comparable manner. The analytical data are given in table III.

The curves plotted from the data in table III are shown in figs. 1 to 4. The curves showing the disappearance of sugars and formation of acids are similar in shape and are typical of bacterial fermentation. The amino-nitrogen curve, which shows the rate of hydrolysis of protein, and the alcohol curve both show the abrupt rise at the beginning which characterizes

TABLE III—SERIES 1: FORMATION OF ACID AND ALCOHOL, DISAPPEARANCE OF SUGARS, AND INCREASE IN AMINO NITROGEN IN SILAGE.

(All data calculated to sample of 100 c. c. of silage juice.)

Age of silage	Total acidity N/10 sol.	Alcohol	NH_2—N.	Total sugars	Total sugars which disappeared
Days	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
0	27.5	0.002	0.069	5.299	0.000
1	47.5	.144	.083	4.934	.365
2	99.7	.065	.089	4.526	.773
3	166.2	.067	.098	2.774	2.525
4	190.0	.082	.089	2.323	2.976
5	233.7	.090	.106	2.078	3.221
6	253.6	.129	.108	1.742	3.557
7	278.3	.095	.109	1.867	3.432
8	226.1	.082	.109	2.203	3.096
12	228.0	.114	.109	2.270	3.029

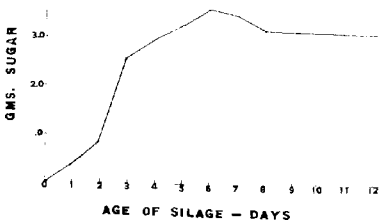

Fig. 1 Curve showing the disappearance of sugars in series 1.

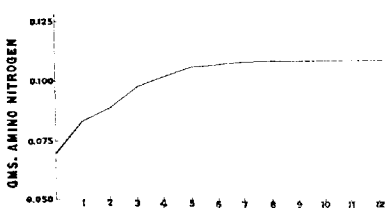

Fig. 3—Curve showing the increase in amino nitrogen (and the rate of hydrolysis of protein) in series 1.

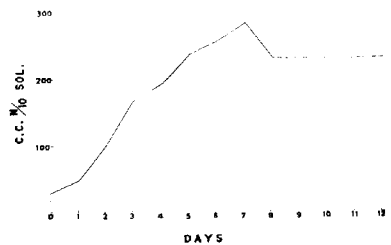

Fig. 2 Curve showing the development of acidity in series 1.

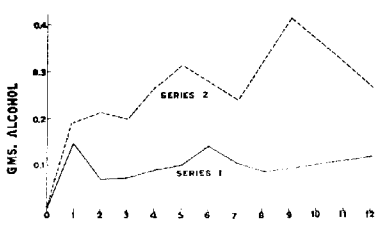

Fig. 4 Curves showing the formation of alcohol in series 1 and 2.

the enzmyic type of curve. However, between the second and third days during the same period in which there appears a marked increase in bacterial activity in the sugar and acid curves, there is also a second rise in NH_3-N curve, which is probable evidence of some proteoclastic action by bacteria.

A similar series of determinations (series 2) made two months later shows a somewhat different set of curves. Field-grown corn at the proper stage of maturity was chopped in the silage cutter, inoculated with material carrying the usual flora of the farm silage cutter, and ensiled as before. In this series total and volatile acid, alcohol, sugars, amino nitrogen, and ammonia nitrogen were determined. The analytical data are shown in table IV.

TABLE IV—SERIES 2: FORMATION OF TOTAL AND VOLATILE ACID AND ALCOHOL, AMINO NITROGEN, AMMONIA NITROGEN AND DISAPPEARANCE OF SUGARS IN SILAGE.

(Data on 100 c. c. of silage juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2-N.	NH_3-N.	Total[1] sugars	Disappear of sugars
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
0	17.3	1.4	0.000	0.021	0.006	3.139	0.000
1	97.0	42.1	.188	.053	.008	1.622	1.517
2	160.3	56.9	.210	.060	.010	1.008	2.131
3	205.4	65.2	.197	.066	.010	.648	2.491
4	235.2	68.4	.262	.095	.026	.235	2.904
5	250.6	68.6	.307	.098	.018	.158	2.981
7	254.4	70.6	.238	.097	.019	.019	3.120
9	263.0	71.0	.403	.104	.021	.019	
13	266.9	78.9	.256	.084	.019	.120	
15	271.6	75.8	.347	.112	.025	.161	
21	291.8	84.0	.354	.128	.027	.....	
30	296.6	79.5	.337	.149	.028	.....	

The form of the acid curves of this series (fig. 5), when compared to the usual form of acid curves, suggests the possibility of so large an inoculation with acid-forming bacteria that the maximum in numbers was reached during the first 24 hours. The curve showing the disappearance of sugars (fig. 6) has the same form. The curves showing the formation of alcohol (fig. 4) is of the enzymic form, like the corresponding curves in series 1. Irregularities in these two alcohol curves

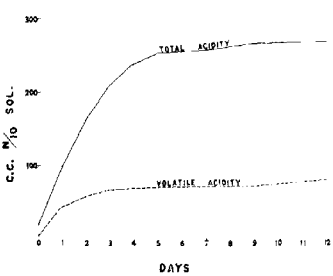

Fig. 5—Curves showing the development of acidity in series 2.

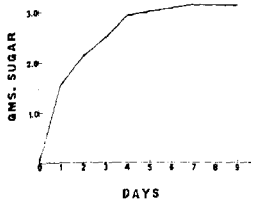

Fig. 6—Curve showing the disappearance of sugars in series 2.

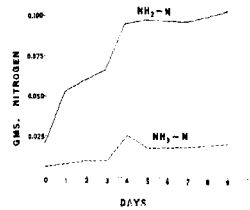

Fig. 7—Curves showing the increase in amino nitrogen and ammonia nitrogen in series 2.

suggest the possibility that each is the resultant of the formation of alcohol by one or more agents and its simultaneous oxidation by other agents. Or there might possibly be some variation between individual samples in the series. The amino-nitrogen curve (fig. 7) shows an evident enzymatic protein hydrolysis during the first three days. Between the third and fourth days, however, an abrupt rise takes place, which bears out the assumption made from series 1, viz, that bacteria figure in the hydrolysis of protein after the first two or three days. It is noteworthy that a similar rise takes place during the same period in the sugar, alcohol, and ammonia-nitrogen curves, indicating a general increase in the activity of microorganims at that time. As previously mentioned, just such a simultaneous rise was noticed in three of the curves in series 1 between the second and third days. This observation lends strength to the evidence in favor of enzymic action in these cases during the first two days.

Corn grown in the greenhouse was used for series 3. This corn made an excellent growth and was practically as good in quality as field-grown corn. The corn was chopped in the laboratory and ensiled as before, but with little opportunity for inoculation. Analytical data on this series are shown in table V. The first jar of silage was opened when only 12 hours old.

TABLE V—SERIES 3: FORMATION OF ACIDS AND ALCOHOLS AND DISAPPEARANCE OF SUGARS.

(Data on 100 c. c. of silage juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	Total sugars	Disappearance of sugars
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*
0	14.0	2.7	0.001	3.850	0
½	25.0	2.8	.079	4.292	— .442
1	36.5	2.9	.123	4.060	— .210
2	39.0	3.0	.272	3.428	+ .422
4	41.5	3.1	.312	2.236	1.614
7	63.5	5.0	.414	1.660	2.190

The increase in the amount of sugars during the first day is interesting, and very probably due to the presence in the corn grain of amylase, which in this case produced sugar from higher carbohydrates faster than the sugar was used. The development of acids is remarbaly small, due perhaps to the slight opportunity for inoculation with acid-forming organisms. It is unfortunate that a larger number of jars was not filled in this experiment so that the later progress of acid formation could have been followed. Since there is no possibility in this series that the formation of acetic acid complicates the question of alcohol formation, some interesting observations are possible. The alcohol curve (fig. 8) after beginning like an enzymic curve takes another abrupt rise between the first and second days, probably when the yeasts become more active. The concomitant production of carbon dioxid in the silage of this series was also measured. (The data are given on page 327.) The carbon dioxid curve (fig. 13) shows the same trend thruout as the alcohol curve, including the rise just mentioned between the first and second days. The carbon dioxid: alcohol ratios show a general increase as the fermentation progresses, as follows:

Age of silage (days)...	½	1	2	4	7
Ratio.....	1:0.51	1:0.61	1:0.86	1:0.79	1:0.97

This increase might be either because the yeasts are taking an increasingly greater part in the fermentation and the ratio therefore approaches the ratio of the ordinary alcoholic fermentation, or because carbon dioxid from other reactions is included in the amount evolved during the first few days. Of course, the above is the result of but a single experiment on this point.

In the next series (series 4) the corn used was from the same greenhouse plot as the preceding. The use of the same tools, etc., a week later, gave opportunity for a much larger inoculation with acid-forming bacteria. This is evidenced by the data shown below. In this series bacteriological counts of some of the samples were made.[1] The technic used was as follows: The sample was ground in a sterile mortar for 15 minutes, 50 gm. weighed out, placed in a liter flask with 500 c. c. of sterile water and shaken 100 times. One c. c. was plated in various dilutions in a yeast-extract agar and incubated for 48 hours at 37° C. Data on this series are given in table VI.

[1]Acknowledgment is gratefully made to Dr. R. E. Buchanan for the use of laboratory facilities and media for making these counts.

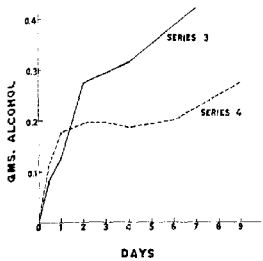

Fig. 8—Curve showing the formation of alcohol in series 3 and 4.

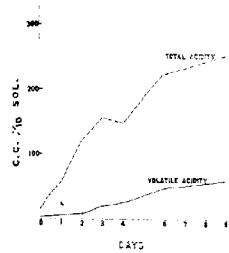

Fig. 9—Curves showing the development of acidity in series 4.

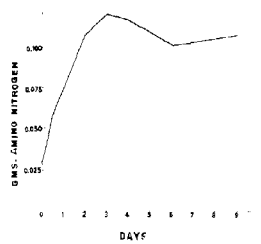

Fig. 10—Curve showing the increase in amino nitrogen in series 4.

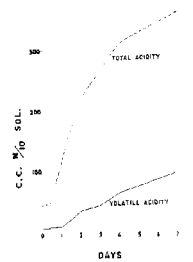

Fig. 11—Curves showing the development of acidity in series 5.

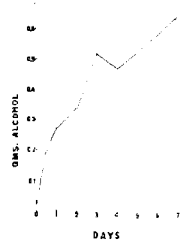

Fig. 12—Curve showing the formation of alcohol in series 5.

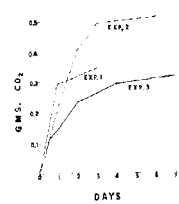

Fig. 13—Curves showing the rate of evolution of carbon dioxide. (Curves 2 and 3 are coincident during the first one-half day.)

TABLE VI—SERIES 4: FORMATION OF ACIDS AND ALCOHOL, AMINO NITROGEN, DISAPPEARANCE OF SUGARS, AND BACTERIAL COUNTS IN SILAGE.

(Data all on 100 c. c. of juice, except bacterial counts.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2-N.	Total sugars	Disappearance of sugars	Bacteria per gram of silage
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	
0	15.5	2.7	0.001	0.028	6.662	0	
½	39.0	3.5	.109	.058	7.036	— .374	9,900,000
1	55.5	4.6	.176	.076	7.594	— .932	
2	121.0	7.1	.192	.108	5.030	(+)1.632	106,500,000
3	156.0	18.9	.193	.120	3.764	2.898	119,000,000
4	148.5	24.5	.182	.116	4.636	2.026	38,800,000
6	223.5	47.8	.197	.101	2.324	4.338	
9	254.0	58.2	.268	.107	2.190	4.472	106,000,000

It should be noted that the preliminary increase in sugars occurs as it did in series 3. The comparatively small bacterial count in the 4-day-old silage is reflected in the total acid, amino nitrogen, and sugar columns. The alcohol curve (fig. 8) shows the same enzymic form as before. The acid curves (fig. 9) show the usual bacterial form. The amino-nitrogen curve (fig. 10) is of the enzymic form as before. The curves showing the disappearance of sugar in series 3 and 4, tho not reproduced here, approach the bacterial form.

A final lot of silage (series 5) was made from corn grown in the fields. This corn lay in the field or on wagons 15 to 20 hours after cutting. It was taken from the farm silage cutter, mixed, and ensiled as before. The data are given in table VII.

TABLE VII—SERIES 5: FORMATION OF ACIDS AND ALCOHOL IN SILAGE.

(Data on 100 c. c. of juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol
Days	*C. c.*	*C. c.*	*Gm.*
0	45.0	6.0	0.022
½	47.5	6.5	.190
1	123.5	7.5	.269
2	218.5	36.8	.342
3	263.0	45.3	.515
4	312.5	65.2	.465
7	362.5	96.8	.633

The initial acid and alcohol content are rather high, as the corn stood so long after cutting. The acid curves (fig. 11) show the usual bacterial form and the alcohol curve (fig. 12) the usual enzymic form, with the later rise presumably due to yeasts.

The data obtained from these five series of experimental silage will be discussed later.

EVOLUTION OF CARBON DIOXID

The amount of carbon dioxid evolved by silage, a constant and characteristic phenomenon of the process, was measured by absorption in caustic potash solution. The silage was packed into cylindrical specimen jars with wide mouths which were fitted with specially made rubber stoppers. A ⅛-inch galvanized-iron pipe was led to the bottom of each jar and the corn was tightly packed around it. This pipe was closed at the top with a rubber tube and a pinchcock. An outlet tube at the top of each jar was connected to an absorption train. The gas was forced thru the train by its own pressure, which was always greatest during the first day, gradually decreasing thereafter. At the end of each period the iron inlet tube of one of the jars was connected to a soda-lime tube and a current of air was drawn thru the absorption train for 30 minutes to remove the carbon dioxid remaining in the jar. The silage could then be removed for analysis. The data from the three experiments are given in table VIII.

TABLE VIII—EVOLUTION OF CARBON DIOXID IN SILAGE.

(All data calculated to sample of 100 gm. of silage.)

Age of silage	Evolution of carbon dioxid		
	Experiment 1 (Gm. CO_2)	Experiment 2 (Gm. CO_2)	Exp'm't 3* (Gm. CO_2)
Days			
½			0.115
1	0.288	0.228	.148
2	(†)	.404	.226
3	.341	.490	
4	(†)	(†)	.262
5	(†)	(†)	
6		.508	
7			.315

*These data are from series 3, p. 323.

The curves plotted from these data are shown in figure 13. All are of the enzymic form, and check with the observations of the writer on all the silage he has made, viz, that the evolution of gas is always greatest during the first day or two, and nearly ceases after about four days. In most cases the rate of evolution is evidently kept up after the first day or two by contributions from bacteria and yeasts. In experiment 3 the curve shows a change of direction and a distinct rise during the second day, coincident, as remarked above, with a similar rise in the alcohol curve in the same experiment (cf. series 3).

RISE IN TEMPERATURE

Another characteristic phenomenon of silage fermentation, but one very much misunderstood in the early days of silage making, is the rise in temperature of the silage. Temperatures

as high as 130° F. have been observed in the silo at or near the surface of the silage. This excessive heating is due to activity of microorganisms greatly accelerated by the presence of atmospheric oxygen, and occurs whenever silage is uncovered and left exposed to the air for a time. The temperature deep in the silo, however, protected from the air and sufficiently removed from the conduction of heat from the surface of the silage, is rarely higher than 80° F. It is rather unsatisfactory to attempt to obtain curves characteristic of bacterial or enzymic fermentation from the rise in temperature of the medium, on account of the number of somewhat extraneous factors involved. The outside temperature is always a factor, and the rise in temperature of the silage might easily affect the rate of chemical reactions or of bacterial growth thus increasing the rate of temperature rise and perhaps changing the nature of the curve. Data which have been obtained from the farm silos[1] and from very carefully insulated laboratory silos suggest that the greater part of the heat developed is due to microbial action. It is considered unnecessary to reproduce these data here as similar data have been published (14). However, one table is subjoined (table IX) showing the rise in temperature at the surface of the silage in one of the farm silos. An iron pipe was forced down into the silage for about 4 feet, and a thermometer, immersed in a test tube full of water, lowered into the pipe so that the bulb was 2 feet below the surface of the silage. The top of the pipe was closed except when the thermometer was pulled up for reading. These data when plotted give a smooth and typical bacterial fermentation curve. Of course, this does not exclude the possibility of some heat production by enzym action.

TABLE IX—RATE OF HEATING AT SURFACE OF SILAGE IN BRICK SILO.

Date	Time	Age of silage	Temperature	Outside temperature
		Days	°F.	F°.
Sept. 19	4 p. m.	0	71.2	64
Do	12 mid.	.....	75.4	
Sept. 20	9 a. m.	.....	82.0	
Do	12 m.	.....	85.6	67
Do	6 p. m.	1	100.8	
Sept. 21	9 a. m.	.....	113.4	
Do	12 m.	.....	115.3	58
Do	4 p. m.	2	117.6	
Sept. 22	9 a. m.	.....	122.2	
Do	6 p. m.	3	123.2	53
Sept. 23	12 m.	4	126.6	52
Sept. 24	12 m.	5	127.2	61
Sept. 25	12 m.	6	128.8	62

[1] With the cooperation of the Agricultural Engineering section of the Iowa station.

DISCUSSION AND CONCLUSIONS

It appears that neither microorganisms nor plant enzyms are alone responsible for the changes which take place in corn silage fermentation. The curves in these pages show that acid production is mainly if not entirely a phenomenon of bacterial activity in the silage. The results from the other experimental silage described above also suggest that the greater part of the acid is produced by microorganisms.

The curves showing the disappearance of sugars are, like the acid curves, generally of the bacterial fermentation type. Altho some of the sugar is undoubtedly changed by enzym action, the greater amount seems to be metabolized by bacteria and yeasts.

The formation of alcohol, however, is evidently a phenomenon primarily of the respiratory or enzymatic activity of the plant cells. This is suggested by investigators in plant physiology, who have often found zymase in plants, and is corroborated by the nature of the alcohol curves shown. The curves suggest a later production of alcohol by yeasts, and results from the other experimental silage support this idea. As stated above, Esten and Mason (6) found large numbers of yeasts in corn silage. Both factors, therefore, probably have a share in the alcohol production.

A similar statement holds good for the hydrolysis of protein as indicated by the amino-nitrogen content of the silage. Proteoclastic enzyms are present in corn grain, and the curves show evidence of their activity. Both the later rise in the amino-nitrogen curves noted above and the results from silage in which the enzyms were destroyed show some proteoclastic activity by microorganisms also. It is noteworthy that E. J. Russell (17) found end products of protein hydrolysis in corn silage made in the presence of toluene, which hydrolysis he ascribed to enzym action.

The evolution of carbon dioxid must be due largely to enzym action. The curves shown all agree on that point, even when the first period was only 12 hours. Evidence that yeasts produce a part of the carbon dioxid after the first day has also been pointed out.

The rise in temperature of the silage is not great except at the surface, where the material is in contact with air. Microorganisms seem to be responsible for most of the heating, but the partial influence of enzyms is not excluded.

SUMMARY

The question of the respective causal relationship of microorganisms and plant cell enzyms to the fermentation of corn silage has long been in controversy. It is difficult to differen

tiate between the activities of these two kinds of agents. Work with antiseptics both by earlier investigators and by the writer is not conclusive. Experimental silage, other than antiseptic silage, has been made, with results of some value; but the most conclusive evidence is obtained by the determination of the rate of change in various phenomena of the fermentation under normal conditions. Curves plotted from these data show that bacteria are mainly responsible for acid production and the concomitant disappearance of sugars. Alcohol is formed first by plant enzyms and later by yeasts. Protein is hydrolyzed first by enzyms and later by microorganisms. Carbon dioxid evolution seems to be very largely due to respiratory or enzymic activities, but yeasts probably have a share in its production after the first day or two. Microorganisms are probably largely responsible for the heating of the silage. Both kinds of factors are always present during silage fermentation and the process is due to the activities of both in the absence of air.

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NITRIFICATION IN ACID SOILS

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NITRIFICATION IN ACID SOILS.[1]

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It is quite generally believed now that nitrification may occur in acid soils, at least to some extent. The addition of lime, however, results in practically all cases in an increased activity of the nitrifying flora. This is especially true when soils are strongly acid in reaction and low in organic matter and when ammonium sulfate is used to measure nitrification.

Probably it is not merely because lime neutralizes acid soil conditions that it increases nitrification. There may be very important indirect effects of liming which are responsible for greater nitrifying action. Lime makes the soil conditions more favorable for the development of the nitrifying organisms and at the same time makes them less satisfactory for what may be called "acid-tolerant organisms" and an entirely different soil flora undoubtedly results. This new soil flora probably has a much less injurious effect on the nitrifying bacteria and may even stimulate their development. There are also many important physical and chemical effects of liming which indirectly affect nitrification. Undoubtedly the influence of lime on nitrification in general represents the combined effects of the material on the chemical, physical and biological soil conditions, all of which are very closely related.

HISTORICAL

The importance of nitrification, which is the making of nitrogenous material available to plants, has led to extended studies of the process, including its relation to the soil reaction. Early workers generally believed that acid soil conditions practically inhibited nitrification. In fact, it was believed that not many bacteria of any kind were active when soils became strongly acid. Even the ammonification occurring in acid soils was attributed to molds which are not so sensitive to acidity as are the bacteria.

Temple (18) found, however, that soils acid to the extent of a 3000 pound lime (CaO) requirement showed an appreciable nitrifying power. He showed also that organic nitrogenous ma-

[1] Acknowledgements are extended to Dr. P. E. Brown for suggestions and assistance in the carrying out of this work and for reading the manuscript.

terials were nitrified much more extensively than a physiologically acid salt, such as ammonium sulfate. It may be suggested here that the explanation for the results leading to this latter conclusion may lie largely in the neutralizing effect of the ammonia which is produced in excess from the organic materials. In other words, the ammonia might be considered to take the place of calcium carbonate in satisfying the demands for a base.

Kelley (13) found that nitrates were not present in Hawaiian soils but calcium carbonate failed to induce nitrification. Evidently acidity was not the sole cause of the lack of the process in those soils.

Hall *et al.* (10) suggest that nitrification does not occur in acid soils except in local areas which are basic. They attribute the injurious effect of soil acidity chiefly to the inhibition of nitrification and of other essential bacteriological processes.

Boussingault and Breal (2) found that forest and meadow soils did not contain nitrates, presumably because of acidity developed in those soils.

Coville (4) concludes that the lack of the proper growth of plants on acid soils is due to nitrogen hunger, which is a result of suspension of the nitrifying process.

White (20) found that nitrification occurred in rather acid soils in Pennsylvania. For example, it took place when the soil showed a lime requirement of 8,373 pounds per acre according to the Veitch method. He suggests the possibility of the organisms becoming adapted to acid conditions. The application of ammonium sulfate to field soils in his experiments led very quickly to the production of acid conditions, the extent of acidity bearing a direct relation to the amount of fertilizer applied.

Fred (6) isolated the nitrifying organisms from Wisconsin soils which showed as high a lime requirement as 20,420 pounds per acre and he found that the cultures of these organisms were able to bring about nitrification when tested in solution. He showed also that organic nitrogenous compounds were nitrified much more rapidly in acid soils than was ammonium sulfate. Acid sands and peats showed a low nitrifying power even when limed, but acid loams permitted of a much more vigorous nitrification when treated with lime. Ammonium sulfate was nitrified more rapidly in the presence of lime, while casein was nitrified faster without it. Furthermore, more organisms were present where lime and organic materials were used and, therefore, greater nitrate assimilation took place under these conditions.

The above results indicate that acid soils not only have a nitrifying flora but that nitrification may occur to an appreciable extent. The work reported in the following pages was planned to throw more light on this problem.

EXPERIMENTAL

The soil used in the experiment was a Carrington loam, loose and friable in texture, and possessing an excellent tilth. It originally showed a lime requirement of nearly three tons of lime per acre, as tested by the modified Tacke method. The organic matter content was somewhat higher than that of average good soil. Previous cropping had shown that the field produced good grain crops but that legumes did not thrive. Just before the soil was sampled for this study, the field had produced a good crop of corn.

Pot studies were carried out in the greenhouse, using four gallon earthenware jars as containers. Lime in various amounts was added to the soils in duplicate pots. Lime was used at the rates of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15 and 20 tons per acre. Moisture conditions were maintained as nearly as possible at the optimum by frequent watering. Samplings were made at intervals of 2, 2, 4, 4, 4, and 7 weeks, covering a period of 23 weeks in all. Samples were taken from the pots by removing the surface soil, mixing the remaining portion, and sampling from the mixed soil. Tests were made for nitrifying power at each sampling. Duplicate 100-gram portions of soil from each of the pots were weighed out in tumblers, 100 mgs. of ammonium sulfate added to each tumbler and the moisture content adjusted to the optimum. The soils were then incubated for 39 days, when the amounts of nitrate present were determined. The lime requirements and the residual carbonates were determined in the soils at each sampling. The modified Tacke method was used for the determination of the lime requirement, and the residual carbonates were determined by decomposing with dilute acid and titrating the carbon dioxide liberated. A second series of pots, 20 in all, one gallon in capacity, were filled with the same soil and the same lime treatments made, except that the 6, 8, 10 and 15 ton quantities were omitted. Alfalfa was grown in these pots to determine the effect of the various applications of lime on this crop. The plants were allowed to grow until they reached the blooming stage. The crop was then harvested from all the pots on the same dates and the weights secured.

NITRIFICATION

For the determination of the nitrates, the Davis (5) modification of the phenoldisulphonic acid method was used. A preliminary test in which a known amount of nitrate was added to the soil showed that the method gave accurate results when rather large amounts of nitrates were present. Nitrate added at the rate of 100 parts of nitrogen per million of soil was recovered completely, as shown in table 1.

TABLE I—THE AMOUNT OF NITRATE RECOVERED FROM TWO SOILS BY THE DAVIS METHOD

Soil	Nitrogen in Orig. Soil	Nitrate added	Nitrogen recovered
	P.P.M.—Nitrogen	As $NaNO_3$ P.P.M.—Nitrogen	P.P.M—Nitrogen
No. 1	2.7	100	103.4
			102.8
No. 1a	2.7		103.3
			102.5
AVERAGE	2.7	100 + 2.7 = 102.7	103.0 error 0.3 percent
No. 2	3.1	100	105.9
			105.9
No. 2a	3.3		106.7
			105.9
AVERAGE	3.2	100 + 3.2 = 103.2	106.1 error 2.9 percent

The amounts of nitrates present in the soils at the various samplings are given in table II.

This table reveals the fact that while there is considerable variation in the results, heavy liming tended in general to reduce slightly the amount of nitrates present in the soils. This was probably due to a greater assimilation of nitrates by bacteria in the presence of lime. It is seen also that in nearly all cases the greatest amounts of nitrates were found at the last

TABLE II—NITRATES IN THE ORIGINAL SOILS AT THE VARIOUS SAMPLINGS

Expressed in parts of Nitrogen per million parts of soil

Treatment	Pots	1st	Av.	2nd	Av.	3rd	Av.	4th	Av.	5th	Av.	6th	Av.	Av. of all
Nothing	A	30.0		19.6		33.9		37.6		47.3		45.9		
	B	27.4	28.7	28.2	23.9	32.1	33.0	36.8	37.2	43.6	45.4	34.8	40.3	34.8
1 ton $CaCO_3$	A	17.4		22.5		30.9		34.6		41.0		28.6		
	B	33.3	25.3	27.8	25.1	29.6	30.2	35.7	35.2	33.0	37.0	44.1	36.4	31.5
2 tons $CaCO_3$	A	36.6		24.5		39.7		34.8		38.9		43.8		
	B	19.7	25.1	25.2	24.9	29.2	34.4	35.4	35.1	33.7	36.3	48.6	46.2	33.7
3 tons $CaCO_3$	A	13.3		19.5		31.1		34.2		29.1		48.8		
	B	16.9	15.1	21.0	20.3	28.8	29.9	34.6	34.4	34.5	31.8	30.5	39.6	28.5
4 tons $CaCO_3$	A	32.1		24.5		35.5		42.2		41.7		46.6		
	B	31.0	31.5	25.2	24.9	28.9	32.2	41.8	42.0	33.4	37.5	37.4	42.0	35.0
5 tons $CaCO_3$	A	25.1		21.2		43.6		40.9		45.8		45.7		
	B	23.7	24.4	25.2	23.2	36.1	39.8	40.6	40.8	40.7	43.7	50.7	48.2	36.7
6 tons $CaCO_3$	A	28.4		27.7		30.3		37.5		38.9		39.2		
	B	33.4	30.9	27.9	27.8	33.3	31.8	37.8	37.6	37.9	38.8	27.9	33.6	33.4
7 tons $CaCO_3$	A	31.8		21.8		33.4		37.4		41.0		34.6		
	B	33.5	34.1	24.5	23.2	28.1	30.7	35.1	36.8	28.1	34.5	41.6	38.1	32.9
8 tons $CaCO_3$	A	15.7		20.7		29.8		35.2		30.6		39.4		
	B	25.4	20.5	27.7	24.2	31.4	30.6	34.8	35.0	44.9	37.8	52.5	45.9	32.4
9 tons $CaCO_3$	A	27.6		24.5		29.2		30.1		29.1		41.3		
	B	27.4	27.5	27.9	26.2	29.6	29.4	39.4	34.7	41.1	35.1	43.1	42.2	32.5
10 tons $CaCO_3$	A	28.2		20.7		27.4		29.8		40.9		42.0		
	B	17.0	22.6	19.7	20.2	26.8	27.1	25.2	27.5	30.6	35.8	44.7	43.3	29.4
12 tons $CaCO_3$	A	23.8		23.3		32.8		36.6		38.5		46.0		
	B	19.1	21.5	18.2	21.2	24.9	28.8	36.3	36.5	39.8	39.1	50.7	48.4	32.6
15 tons $CaCO_3$	A	21.0		23.5		20.4		24.2		33.4		45.5		
	B	19.8	20.4	23.3	23.4	25.5	22.9	35.6	29.9	39.0	36.2	34.9	40.2	28.8
20 tons $CaCO_3$	A	13.5		21.8		25.4		32.4		27.0		41.6		
	B	27.2	20.4	25.4	23.6	28.9	27.1	34.4	33.4	31.8	29.4	44.8	43.2	29.5
													Average	32.5

sampling. The greenhouse conditions were evidently favorable for the nitrifying process. The addition of large quantities of lime apparently did not increase the nitrification of the organic matter in the soil. Apparently lime should not, therefore, cause any marked loss of organic matter from normal soils, provided it is applied as the carbonate. The increase in numbers of organisms and their assimilation of nitrates may in fact tend to reduce the losses by leaching.

Examining table III, it appears that there were greater irregularities in the nitrification of ammonium sulfate in the soil in tumblers than were found in the original soil in the pots. Here, however, lime increased nitrification considerably. Three and four tons of the carbonate doubled the amount of nitrates produced and there was some increase in the production of nitrate with the increasing amount of lime, even up to the 20 ton treatment. This would indicate that a non-acid soil, or possibly one containing considerable carbonates, might respond to lime. To what the stimulative effect of such large amounts of lime may be due cannot be definitely stated. It is possible that each lime particle may represent local points of nitrification. If this were true, the greater application would supply a larger number of nitrifying centers and, therefore, a somewhat greater aggregate nitrification. This is in accord with the results of other workers who have shown that increased fineness of division of the limestone used caused greater nitrification.

Evidently the neutralization of the original soil acids is not the only factor to consider, for a comparatively small quantity of lime is sufficient for that purpose.

With larger applications than six to seven tons of lime, increases in the amount of nitrate produced were much less marked, and with additions of nine and ten tons nearly as much nitrate was produced as when the application was doubled. There is, evidently, a point beyond which greater additions of lime have little or no effect on nitrification.

Taking the average of all samples shown in table II, the maximum amount of nitrates was found in the original soil with the four and five ton applications of lime. A rather consistent increase is also shown which each successive sampling in the soils under various treatments. This is doubtless due to the increasingly favorable conditions for nitrification. In the tumbler experiments, averaging the results with all treatments, there was an increase in nitrates at each of the first three successive samplings, followed by a drop at the next two and an increase again at the last. These fluctuations may be without significance, but it is possible that they are partially the result of the effect of the lime on the bacterial species relationships.

The averages given at the bottom and in the right column of the table represent a considerable number of determinations and should be of more significance than the individual figures.

TABLE III—NITRIFICATION OF $(NH_4)_2SO_4$ IN SAMPLES DRAWN AT INTERVALS OF 2-2-4-4-4 AND 7 WEEKS

		SAMPLINGS						
Treatment	Pots	1st	2nd	3rd	4th	5th	6th	General Aver.
Check	A	Lost	69.8	79.4	81.4	77.6	67.5	75.0
		64.8	68.2	79.6	92.0	95.5	63.6	
	B	67.6	59.2	81.6	72.4	75.0	91.0	
		44.6	71.7	81.1	93.2	75.9	89.9	
1 ton $CaCO_3$	A	88.2	67.7	80.2	93.0	78.8	108.6	86.5
		57.0	95.3	80.6	80.9	86.0	108.6	
	B	104.1	82.2	102.5	102.8	107.6	76.7	
		77.8	90.0	98.6	95.5	107.8	95.4	
2 tons $CaCO_3$	A	103.5	68.3	132.6	77.2	98.8	123.1	118.3
		89.4	124.9	101.8	154.3	113.8	99.5	
	B	125.6	117.7	120.3	101.1	123.0	141.9	
		171.3	120.7	122.0	155.6	123.1	127.1	
3 tons $CaCO_3$	A	146.0	147.2	147.8	104.2	125.7	143.9	143.7
		126.8	139.4	154.3	158.7	142.5	134.6	
	B	129.9	145.2	150.4	156.2	146.1	162.3	
		46.7	137.5	161.5	152.2	134.6	155.5	
4 tons $CaCO_3$	A	154.5	175.0	178.9	128.7	Lost	148.5	157.8
		152.1	175.3	165.7	158.6	172.0	156.8	
	B	129.3	156.0	174.2	166.2	103.5	164.4	
		161.6	168.3	155.5	171.1	158.3	173.2	
5 tons $CaCO_3$	A	162.8	160.6	172.9	143.1	114.6	194.9	165.5
		161.5	200.0	156.3	172.3	143.4	168.9	
	B	164.2	164.4	177.9	170.6	123.4	181.1	
		162.9	184.4	176.3	168.7	169.7	182.4	
6 tons $CaCO_3$	A	178.8	161.4	194.4	175.5	160.2	165.4	171.4
		156.2	192.4	164.9	Lost	160.2	197.3	
	B	161.1	156.9	174.2	156.6	152.1	191.8	
		159.2	184.0	162.9	182.6	158.3	204.3	
7 tons $CaCO_3$	A	172.4	190.1	192.7	170.4	182.7	179.3	180.9
		157.8	199.9	172.0	189.7	179.4	195.5	
	B	173.1	173.0	199.9	177.0	178.9	181.9	
		162.5	184.7	177.5	172.8	178.9	199.0	
8 tons $CaCO_3$	A	199.9	176.1	181.6	168.1	181.9	186.4	182.1
		191.5	198.6	196.0	166.2	185.4	208.9	
	B	181.8	157.9	199.8	170.1	155.1	195.5	
		181.8	178.5	190.0	161.3	180.0	174.5	
9 tons $CaCO_3$	A	179.6	177.1	187.3	168.8	182.0	187.1	181.6
		179.6	192.8	191.0	168.5	195.9	179.3	
	B	177.2	161.2	192.9	165.5	174.2	179.0	
		188.2	192.3	203.2	184.3	172.1	180.0	
10 tons $CaCO_3$	A	202.4	169.2	196.6	150.2	184.2	187.0	188.0
		181.9	202.3	192.7	195.0	180.9	182.8	
	B	211.9	182.5	187.2	167.8	178.6	184.4	
		187.4	202.4	198.8	199.6	183.5	193.3	
12 tons $CaCO_3$	A	206.8	182.9	181.4	186.1	174.3	182.9	189.0
		184.8	199.4	197.1	178.7	174.3	190.9	
	B	212.5	177.5	194.7	185.8	189.5	192.0	
		180.9	205.0	211.4	174.3	183.7	189.4	
15 tons $CaCO_3$	A	209.6	197.8	215.4	188.0	182.5	187.6	191.6
		196.5	197.7	210.1	171.6	185.1	191.3	
	B	197.4	188.9	198.3	180.4	183.8	191.8	
		193.0	196.0	204.5	161.5	187.2	178.6	
20 tons $CaCO_3$	A	217.1	212.5	224.4	190.7	187.0	197.5	193.1
		196.5	199.4	217.8	185.4	186.0	182.1	
	B	199.0	197.6	222.3	172.3	Lost	182.8	
		195.9	197.6	209.5	172.4	145.5	191.1	
Average		156.1	160.2	167.3	155.0	151.8	163.5	155.3

WHY DO ACID SOILS NITRIFY?

Recent theories of the relation of the process of nitrification to acid soil conditions have been considerably modified. The nature of soil acidity is better understood and it is now believed that the concentration of the hydrogen-ion is the fundamental cause of acidity. The presence of acid salts, relatively insoluble acids and amphoteric substances, may cause the amount of base taken up by the soil to be high, when there is really only a small concentration of active acids. Potential acidity and the hydrogen-ion concentration of the soil are certainly widely different at times, and this difference may well occur in most cases. There is undoubtedly a difference in the effect of active acidity and potential acidity on nitrification.

Furthermore, nitrates are nearly always found to some extent even in rather acid soils. It is possible that the nitrifying organisms may be more resistant than has been believed formerly. Probably also other bases than carbonates function locally and permit nitrification to occur to some extent.

Molds have been found to be rather efficient ammonifiers and these organisms endure extremely acid conditions. Practically no soil, therefore, should be too acid for ammonification to occur. When ammonia is produced, acidity is neutralized and at each point of production of ammonia, nitrification may begin. Any ordinarily acid soil may therefore be expected to contain the nitrifying organisms and to nitrify to a limited extent at least. The reasons given also explain why organic materials such as casein may nitrify more readily in acid soils. That is, the ammonia produced is removed so rapidly by the soil acids that a toxic accumulation cannot occur as soon as may be the case in the limed soils. The neutralization of the acidity by the ammonia gives also a more suitable reaction for the functioning of the nitrate organisms. There are doubtless other factors to consider, however, for some nitrogenous organic materials have been found to be nitrified more readily in the presence of lime. Ammonium sulfate always nitrifies more readily when lime is supplied, but here there is the double effect of the sulfuric acid liberated and of the nitric acid produced.

It is possible, too, that organisms may endure a greater acidity in soils than in culture media. The colloidal materials of the soil, such as the proteins, acting as buffers, serve to protect the organisms and they also supply food for the bacteria.

The common method of reporting soil acidity is in terms of lime requirement, rather than in hydrogen-ion concentration, which represents the active acidity. The hydrogen electrode is the most reliable method yet developed for determining the hydrogen-ion in soils, but it has not been used extensively. In a few instances, acidity has been reported in terms of normality,

regardless of hydrogen-ion concentration. Hall, Miller, and Gimingham (10) report easily soluble acids extractable by washing, equivalent to 1/60th to 1/140th normal. When this is compared with n/10 hydrochloric acid, which is about 90 per cent ionized and gives a hydrogen-ion concentration of nearly $9x10^{-2}$ gm. ions per liter, it seems very probable that the soil acid extracted, which was equivalent to 1/6 to 1/14 as great a normality, gave a very much lower hydrogen-ion concentration than that theoretically possible, probably lower than that of acetic acid which is about 1/70th as great as for hydrochloric acid of the same concentration. On the basis of the ionization of acetic acid, the 1/60th normal extraction should have represented a hydrogen-ion concentration in the soils of approximately $2.4x10^{-4}$ gm. ions of hydrogen. This would represent a rather acid soil, while on the basis of the ionization of hydrochloric acid the concentration should be about $1.5x10^{-2}$, which is more acid than soils probably become. Such an extracted acidity would perhaps be the result of acids, some fairly strong, others very weak. While the weak acids could be titrated, they might be completely suppressed from ionization by the stronger acids.

The hydrogen-ion concentration found by Sharp and Hoagland (16,12) varies from $2x10^{-4}$ in very acid soils to $2x10^{-10}$ in alkaline soils. Plummer (15), in studying the film of hygroscopic water in the soil, where the soil organisms are generally active, found that the hydrogen-ion concentration there was somewhat greater than in the soil suspension in the case of acid soils, while in basic soils the hydroxyl-ion concentration was greater. Considering all available data along this line, it would seem that the soil acidity usually reported is much larger than that which would be represented by an equivalent in hydrogen-ion concentration, and the acidity of the soil film is not often determined.

The acidity found in very acid soils, as suggested above, is about 1/450 that of n/10 hydrochloric acid, which is considered a dilute solution. But many soil organisms are doubtless killed or retarded by even a much weaker acidity than this. Reports given by several workers (1, 4, 6, 7) have shown many organisms to be sensitive to hydrogen-ion concentrations of 10^{-5} and 10^{-6}. Gruzit (9) found that the general flora of the soil solution from a sand culture was subjected to a germicidal toxicity by an acid concentration of n/1200 when sulfuric and hydrochloric acids were used. A concentration of n/2164 acids caused the destruction and increase of bacteria to balance each other. This latter acidity is roughly equivalent to a $4x10^{-4}$ hydrogen-ion concentration, which is very close to the acidity of the more acid soils. The same worker found also that the toxic limits for corn seedlings, when the above acids were used, resulted in a 43 per-

cent reduction in the number of soil bacteria present. This data would indicate that acidity may cause a direct injury both upon plants and upon organisms, and that the extent of the injury depends in part at least upon the hydrogen-ion concentration. A discussion of the toxic hydrogen-ion concentrations in relation to various organisms may be found in a publication by Fred (6).

There can be little question but that certain very important organisms are quite sensitive to acidity. Thus it is reported by Gainey (8) that azotobacter were not found in soils with a hydrogen-ion concentration of 10^{-6}. This is only a mildly acid soil and therefore it would seem that the solution of the nitrogen problem, by increasing azofication, is dependent very largely upon the reaction of the soil. The nitrifying organisms are also quite sensitive to acidity, but it is always possible for such organisms to be active in local areas of slight acidity. It may be possible also that chemical actions occurring within the soil films are modified by osmotic and surface energy in such a way that organisms are not as readily affected as under less natural conditions. There is data to show that densities are modified appreciably (as much as 0.5 percent) when substances are sufficiently finely divided and that increasing the fineness of division greatly increases solubility. Since all these factors and many others are operative in soils, it is not possible to determine the exact environment to which soil organisms are subjected. The organisms themselves have effects that cannot be measured. The carbon dioxide which they produce increases the surface energy of the soil films, while the organic substances which they help put into solution lower the surface energy. The death of organisms is brought about by chemical actions upon the protoplasm of the organism. The chemical energy within the soil film water, expressing itself in certain reactions, is much modified by the surface energy of the film and hence there is a close but indefinite relationship between the toxicity of the soil acids and their various effects.

A few facts stand out prominently in the above discussion. First, a soil may be theoretically too acid for nitrification and yet it is very seldom that the organisms and some nitrates are not present. The smallest quantities of nitrates are usually found in water-logged soils where aeration would be a limiting factor for the nitrifying organisms. Second, several tests have shown an appreciable nitrification in soils of very high acidity. As high as 10,000 and even 20,000 pounds lime requirement have been reported when nitrates were present. Soils also which contained scarcely any nitrate, presumably on account of acidity, were found to nitrify in solution, proving the presence of the organisms.

ARTIFICIAL ACIDITY

The following study of nitrification in soils made acid by additions of sulfuric acid was carried out in tumblers. Two soils were used, both slightly acid at the start, and made strongly acid by adding varying amounts of acid. One soil was of the same type, tho not from the same field, as the soil used in the pot studies. The other was quite sandy and low in organic matter. It is probable that sulfuric acid should give a fairly high hydrogen-ion concentration in the soil and that its toxicity would be due to this ion rather than to the sulfate ion. A higher hydrogen-ion concentration should be produced with the sandy soil, because of lack of organic matter and consequent lack of buffering. Therefore, the acid should be more toxic on the sandy soil. Below are the results showing the lime requirement by the modified Tacke method (17), since no method was available for the determination of hydrogen-ion concentration.

Table IV shows that the results were as predicted. The sandy soil was more severely affected by a similar addition of sulfuric acid. Both soils show a wonderful response to lime, but considering the lower nitrifying power, the sandy soil really shows the greater effect. The water extract of all these acid-treated soils showed a high degree of acidity by the litmus test and also by the color given to methyl orange, which is proof that there was no reaction by which the acid was removed from active functioning by the soil. It is quite remarkable that the loam showed considerable nitrates present with as high a lime requirement as twelve tons, while the other soil showed little with six and a half tons. These figures indicate that the statement that a soil has a lime requirement of 5 to 10 tons has little significance. Nearly twice as much nitrate is present with a 12 ton requirement on

TABLE IV—SHOWING ACIDITY AND NITRATES AFTER FIVE WEEKS' INCUBATION

Loam Soil	Acidity Tons	P.P.M. Nitrate Nitrogen
Soil alone	4.20	97.1 99.7
Limed	0.90	201.6 225.9
H_2SO_4 added	5.70	78.1 72.7
H_2SO_4 added	7.10	55.1 52.1
H_2SO_4 added	8.55	46.9 49.3
H_2SO_4 added	10.90	37.9 31.2
H_2SO_4 added	12.90	33.1 34.7
H_2SO_4 added	20.40	None

Sandy Soil	Acidity Tons	P.P.M. Nitrate Nitrogen
Soil alone	2.85	66.5 59.5
Limed	0.20	208.3 227.3
H_2SO_4 added	4.50	62.5 24.8
H_2SO_4 added	5.10	18.4 19.5
H_2SO_4 added	6.50	8.9 6.2
H_2SO_4 added	9.95	Trace
H_2SO_4 added	11.05	Trace
H_2SO_4 added	19.90	None

the loam as with a 5 ton requirement on the sandy soil. On both soils, however, the decrease in nitrates follows quite regularly the increase in acidity.

A second study was made to determine what effect lime and acid might have upon a soil which was only slightly acid. The test was made with ammonium sulfate and the tumblers were incubated 42 days. The results are shown in the table V.

The test shows the same general result. Lime increased nitrification very markedly and the larger application brought about the greater increase. Again nitrification occurred in the presence of the added sulfuric acid, but only to a slight extent.

In table VI appear the results of nitrification tests on different soils. The object of this experiment was to determine whether an increase in nitrification would result from liming a soil already containing carbonates. On one soil casein was used to test for nitrification, while on the other two ammonium sulfate was employed. One acid soil was tested by the use of casein, one lime treatment being made. For the nitrification of ammonium sulfate, soils rich in carbonates were used, but lime was applied also.

The above data indicate that it is possible to add sufficient acid to check ammonification, but nitrification ceases where a light application of phosphoric acid is made. In this test it may be observed that lime has checked both ammonification and nitrification, where casein is used. On the soils containing the large amounts of natural carbonates, the application of lime increased nitrification, but only to a small extent. If a soil were only neutral or contained just a small excess of carbonate, it might be expected that nitrification would be stimulated by liming. Such a soil was not available, however, for use in this work. Since the nitrifying organisms are very sensitive to acidity, some basic material must be present before nitrification can occur. With only a small amount of base the supply is soon exhausted and nitrification is sooner checked than when a larger amount is present. The more lime and the more finely it is divided, therefore, the greater is the nitrification in the soil, up to a certain limit. This limit would doubtless vary for different soils, but it is probably several tons per acre in most cases.

TABLE V—THE EFFECT OF LIME AND ACIDS UPON NITRIFICATION

Treatment	Total Acidity Tons	P.P.M. Nitrates as Nitrogen
Soil alone	2.3	30.3
Soil + 10 tons $CaCO_3$	....	151.1
Soil + 20 tons $CaCO_3$	....	153.8
Soil + H_2SO_4	9.2	Trace
Soil + H_2SO_4	16.2	Trace
Soil + H_2SO_4	60.2	None

TABLE VI—NITRIFICATION IN VARIOUSLY TREATED SOILS, INCUBATED 43 DAYS

Treatment	Tons Acidity	P.P.M. Ammonia N.	P.P.M. Nitrate N.
Soil + Casein	1	772.8	176.4
Soil + Casein + Lime	10	649.6	62.5
Soil + Casein + H_3PO_4	4.8	817.6	Trace
Soil + Casein + H_3PO_4	24.7	84.0	Trace
Soil + Casein + H_2SO_4	20.6	117.6	Trace
Soil with 50 tons natural carbonates + $(NH_4)_2SO_4$	...	....	120.0
Soil with 50 tons natural carbonates + 10 tons $CaCO_3$ + $(NH_4)_2SO_4$	...	....	134.4
Soil with 50 tons natural carbonates + 20 tons $CaCO_3$ + $(NH_4)_2SO_4$	...	....	120.0
Soil with 15 tons natural carbonates, alone + $(NH_4)_2SO_4$	...	....	147.3
Soil with 15 tons natural carbonates + 10 tons $CaCO_3$	...	....	176.4
Soil with 15 tons natural carbonates + 20 tons $CaCO_3$	...	....	176.4

SOIL ALKALINITY

The question whether a soil may be too alkaline for bacterial life is of considerable interest. Quick-lime is sometimes employed for partial sterilization and evidently restricts bacterial growth. Calcium carbonate, however, probably acts very differently from quick-lime. The concentration of soluble salts which is commonly termed alkali is not under consideration here. Such conditions would undoubtedly retard or completely inhibit normal bacterial activity. Hoagland has reported that in nutrient solutions, an equal divergence of the hydroxyl-ion concentration from the neutral point caused greater toxicity than did an abnormally high hydrogen-ion concentration, when barley seedlings were grown. The response of organisms to variations in reaction would not be identical, but might be very similar. A hydroxyl-ion concentration of 2.5×10^{-5} was distinctly toxic, while a hydrogen-ion concentration of 0.7×10^{-5} was favorable to growth. Only when the hydrogen-ion concentration was 3×10^{-4} did serious toxicity occur.

In recent work by Hoagland and Christie (11), a hydroxyl-ion concentration as low as $10^{-2.76}$ is reported where calcium oxide (0.5 per cent) was added to the soil. This is very alkaline and tho possible to produce, does not occur, it may safely be said under field conditions. The same workers found that calcium carbonate added to the soil gave an alkinity of 10^{-6}, which is perhaps close to toxicity, but this concentration did not endure for long and would probably cause no injury. The quicklime treatment, however, did cause sterilization and no nitrification occurred. It may be mentioned also that Gruzit (9) found an alkalinity of 10^{-4} somewhat injurious to organisms in sand cultures. The

effect of lime in general, however, seems to depend very much upon the soil. After a few days, any alkalinity produced by lime has usually become much less marked and has continued to become smaller as time elapsed. Plummer (15) reports that calcium carbonate may increase the alkalinity, but not to an injurious extent. In this work heavy applications have been made, but the addition of 20 tons to soil already containing 50 tons of carbonate did not depress nitrification. The results from the alfalfa grown on the pots likewise would not warrant any general statement of toxicity.

Considering all the data, it may safely be said that practically never would a toxic alkalinity be produced by liming, especially with calcium carbonate.

ACIDITY RESULTS

The acidity changes are given in table VII, as indicated by the modified Tacke (17) method. Determinations were not made at all samplings, since the amount of lime requirement indicated had become nearly constant in some cases and the data was therefore not essential.

Examining table VII, it appears that the lime requirement of the soil was about three tons, the average of the five tests being 2.75 tons. The addition of lime up to 4 tons per acre diminished the acidity. From 4 tons on, a practically constant acidity is shown. The variations which occur at different samplings and with different determinations are not large, considering the many opportunities for errors in sampling the soil. The table shows also that there was very little difference in the lime requirement at the third and fifth samplings, so that no information was lost by failing to run tests at the fourth sampling. The reaction

TABLE VII—LIME REQUIREMENT IN TONS OF CALCIUM CARBONATE PER ACRE, AT THE VARIOUS SAMPLINGS [1]

Treatment	1	2	3	5	6	Aver.
0	2.80	2.80	2.70	2.65	2.70	2.75
1 ton $CaCO_3$	2.40	2.45	2.15	2.05	2.50	2.31
2 tons $CaCO_3$	1.15	1.05	1.05	1.35	1.30	1.38
3 tons $CaCO_3$	1.00	0.90	0.70	1.05	0.70	0.87
4 tons $CaCO_3$	0.75	0.80	0.75	0.87	0.50	0.73
5 tons $CaCO_3$	0.85	0.70	...	0.75	0.45	0.55
6 tons $CaCO_3$	0.80	0.65	...	0.80	0.40	0.53
7 tons $CaCO_3$	0.70	0.70	0.75	0.65	0.55	0.67
8 tons $CaCO_3$	0.75	0.75	0.65	0.65	0.45	0.53
9 tons $CaCO_3$	0.90	0.70	0.60	0.60	0.55	0.67
10 tons $CaCO_3$	0.95	0.75	...	0.60	...	0.57
12 tons $CaCO_3$	0.95	0.70	...	...	0.40	0.68
15 tons $CaCO_3$	0.85	0.75	0.75	...	0.65	0.75
20 tons $CaCO_3$	0.80	0.75	0.80	...	0.50	0.71
Average from 4 tons down	0.83	0.73	0.71	0.70	0.50	0.64

[1] The lime requirement was not determined at the fourth sampling.

between the lime and the soil acids has evidently taken place rather rapidly. It may be presumed that the more reactive acids were neutralized at the end of two weeks. From that time on there was a gradual using up of base. The entire test covered 23 weeks. During the last seven weeks, 400 pounds of carbonate were apparently used up.

The question may arise why the method showed a lime requirement in all cases. The answer is that soils do not react under field conditions to permit complete neutralization. This is due to the fact that soils are not thoroly enough mixed to secure intimate contact and that slowly soluble acids develop locally either by hydrolytic processes or otherwise. Decay naturally produces some acidity, at least temporarily. Then, too, there must be an entire absence of lime at local points until the soil and lime have been for a long time in contact. The results show that after a long contact only half a ton of acidity remained, regardless of whether little more than enough lime was supplied to neutralize the soil acids, or whether a large excess was supplied. Of course, when the soil is brought into intimate contact with carbonate in the shaking machine, more complete neutralization would be expected, and tests show that it occurs.

As proof of the above suggestions, tests were made with the Truog (19) qualitative test. It was found that in all tests, the soils receiving a two ton application of lime gave a slight indication of acidity, while from three tons on the reaction was basic.

This shows again that three tons of lime, the amount indicated by the modified Tacke method, was sufficient to neutralize the active acidity of the soil. The qualitative test evidently shows when sufficient lime has been added to satisfy the more active acidity, regardless of whether or not complete neutralization has yet occurred.

RESIDUAL CARBONATES

In table VIII are given the results of the determinations of residual carbonates in the variously treated soils in the greenhouse. The carbonates were determined by decomposing with phosphoric acid (1-15) and collecting the carbon dioxide evolved.

A study of the table reveals the fact that there was a gradual using up of carbonates up to the last sampling, but that by far the greatest demand came at once. The averages are taken for all treatments above three tons, since that was approximately the lime requirement of the soil. There has been, therefore, a very rapid neutralization of the active acids. The method indicates a lime requirement of 2.75 tons. After deducting the residual carbonates from the original application, it is found that 2.8 tons, almost identically the average lime requirement of the untreated soil, have been used in two weeks by the soil. As the qualitative test indicates that this amount has neutralized the

TABLE VIII—RESIDUAL CARBONATES IN TONS OF $CaCO_3$

Treatment	1st Sampling	2nd Sampling	3rd Sampling	5th Sampling	6th Sampling	Amt. $CaCO_3$ used up 1st Sampling	Amt. $CaCO_3$ used up 6th Sampling
1 ton	0.15	0.05	0.00	0.00	0.00	0.85	1.00
2 tons	Lost	0.20	0.30	0.30	0.00	Lost	2.00
3 tons	0.95	0.70	0.55	0.25	0.10	2.05	2.90
4 tons	1.45	2.25	1.25	0.70	0.15	2.55	3.85
5 tons	2.75	2.00	2.10	1.60	0.70	2.25	4.30
6 tons	3.15	2.30	2.80	2.85	1.40	2.85	4.60
7 tons	4.20	3.60	3.25	3.35	2.65	2.80	4.35
8 tons	5.60	4.55	4.20	3.90	3.30	2.40	4.70
9 tons	6.50	5.80	5.90	4.55	4.00	2.50	5.00
10 tons	6.80	6.45	5.70	5.80	5.55	3.20	4.45
12 tons	9.50	7.95	7.50	7.10	6.60	2.50	5.40
15 tons	11.90	10.25	11.50	10.60	11.10	3.10	3.90
20 tons	16.45	16.10	15.10	14.80	14.20	3.55	5.80
Aver.	6.80	6.10	5.90	5.60	5.00	2.80	4.60

soil acids, and as enough determinations have been made to remove most of the possibilities of error, it seems evident that the active acids have been correctly estimated and that a three ton application of lime would be an abundance for this soil.

The next question that arises is, what became of the excess carbonate which was used up as the experiment proceeded. The data shows that during the second two weeks an additional 1,400 pounds were used; during the four weeks following, 400 pounds more; during the succeeding eight weeks an additional 600 pounds and finally, during the last seven weeks, 1200 pounds were used up by the soil, until there is a total of a little more than 4½ tons used. Part of the high figures are doubtless due to errors in determining such very large amounts of carbonates. There is no doubt, however, but that the soil has used a considerable excess of carbonate over the indicated needs. The reason for this probably is that very insoluble and little ionized acids have gradually reacted. Furthermore, the nitrogen changes which have occurred have made increased, tho small, demands for base, up to the last sampling. And some organic acids must have developed while the process of nitrification has been occurring and these would use some of the base temporarily. Still another cause for the disappearance of base is its fixation by the soil mineral complexes and thus, to a limited extent, its change to silicates (14) which were not reactive with the phosphoric acid used in determining the carbonates. The data indicates that soils may undoubtedly use carbonate in considerable excess of the demands for neutralization of active acidity.

BACTERIA IN THE SOILS

In this study only very limited bacteriological examinations were made, and not a great significance attends the results. The

TABLE IX—THE NUMBER OF ORGANISMS IN MILLIONS PER GRAM ON GELATIN PLATES

Treatment	SAMPLINGS			
	1st	2nd	3rd	4th
Check	1.12	2.86	4.76	4.76
1 ton $CaCO_3$	2.02			
2 tons $CaCO_3$	2.53			
3 tons $CaCO_3$	2.93			
5 tons $CaCO_3$	2.48	3.84	...	5.06
6 tons $CaCO_3$	3.57			
7 tons $CaCO_3$	2.68			
9 tons $CaCO_3$	2.62			
10 tons $CaCO_3$	2.52			
12 tons $CaCO_3$	3.00			
20 tons $CaCO_3$	3.14	3.94	5.80	4.56

gelatin medium used probably does not permit of the development of any of the nitrifiers. Nevertheless, many of the organisms which are plated cause ammonification and this process must precede nitrification, so that undoubtedly there is an indirect relationship. Large numbers of organisms mean greater competition and vice versa, and this is a factor in any activity.

In this study only a few platings were made, mainly at the first sampling and then at various later intervals, primarily to determine what effect the lime had upon numbers. The results are given in table IX.

In general the effect of the lime was to increase the number of organisms. The effect was more marked at the first sampling than later. The number of organisms increased proportionately more without the lime than with it, and has more than doubled in many cases. The increase is due doubtless to favorable conditions of moisture and temperature. Evidently the acidity of such a degree as occurs in this soil is not very toxic to common soil organisms. This is probably more especially true of soils such as this one, which is relatively high in organic matter and in good physical condition.

POT STUDIES WITH ALFALFA

The pot studies with alfalfa were rather inconclusive. The pots were treated in the same way as for the bacteriological studies and alfalfa was seeded thickly. By successive thinning the number of plants per pot was finally reduced to five. This selection largely eliminated differences due to individuality of plants, and should permit each treatment to exhibit its maximum production.

All plants were thrifty in appearance and growth, and it was not possible to detect any difference until toward the end of the experiment, when some of the treatments seemed to forge ahead. The plants were harvested soon after blooming started and both green and dry weight determined. A summary is given in table X.

TABLE X—GREEN AND DRY WEIGHTS OF ALFALFA

	Treatment—Tons $CaCO_3$									
Green Weight	0	1	2	3	4	5	7	9	12	20
	gms	gms	gms	gms	gms	gms	gms	gms	gms	gms
Pot 1	14.0	19.5	21.0	24.0	16.0	20.0	14.0	19.0	18.0	19.0
Pot 2	18.0	22.0	24.0	32.5	13.0	19.0	13.0	20.5	16.0	12.5
Dry Weight										
Pot 1	4.0	5.5	5.8	6.6	4.3	5.5	3.7	5.3	5.0	5.2
Pot 2	5.2	6.2	6.8	8.2	4.9	5.0	3.3	5.5	4.6	4.0

The table shows that in a general way the dry weights follow the same tendency as the green weights, but the effects are rather more marked. The maximum weight is produced with the three ton treatment, which is the lime requirement of the soil. The weight produced seems to increase gradually up to this point, after which there is a drop and no treatment in excess of this amount has produced much greater growth than the untreated soil. These results would indicate that too much lime might be just as injurious as not enough, but the data is too limited for definite conclusions.

The pots with the three ton treatment also bloomed first. In fact many of the others did not bloom for several days after. The pots were examined for nodules, which were found in considerable abundance, so that differences were not due to lack of inoculation. A second crop was allowed to mature and produce seed, but was not harvested. The same difference in rate of growth and time of blooming was noted as before. A considerable setting of seed occurred on the unlimed as well as on the limed pots and, in general, the second crop behaved in the same way as the first.

CONCLUSION

The results of this study give further proof of the capacity of acid soils for nitrification. Even when most of the soil contains a toxic hydrogen-ion concentration, there may be local areas of low intensity where nitrification can occur. It is usually true that soils reported highly acid contain a relatively low concentration of active acidity. Highly soluble acids which would be very active leach too readily to accumulate in soils, except as salts. Slowly soluble acids and amphoteric substances may cause a soil to show a high lime requirement and yet not produce a highly toxic acidity. An illustration of this is found when 4 to 5 tons of sulfuric acid added to the soil nearly stopped nitrification, while naturally acid soil of a higher lime requirement nitrified very readily.

The results of this work indicate that tho very large amounts of lime may give greater nitrification, only that which is necessary to neutralize the most active acids is essential for adequate nitrification and maximum crop production. Too rapid a nitrification is a wasteful process, because neither plants nor or-

ganisms can assimilate the nitrates as fast as produced. Fred and Graul (6) found in their work that only half the application of lime indicated by the Truog barium hydroxide method was necessary. The desired end is, of course, to stimulate nitrification only sufficiently to meet the needs for maximum crop production.

SUMMARY

1. Nitrification has been found to occur in the presence of a rather high lime requirement.

2. A scarcely measurable effect was produced in the nitrification of the original soil nitrogen, by the application of calcium carbonate. Lime did cause a marked increase, however, in the nitrification of ammonium sulfate.

3. The soil acids were never completely neutralized, even with the very heavy applications of lime, apparently because of the slow solubility of a part of the acidity present.

4. The amount of carbonate taken up by the soil, calculated upon the basis of residual carbonates, agreed well with the indicated lime requirement. After several weeks, however, more lime had been taken up than was equivalent to the requirement according to the modified Tacke method. This was because the method, permitting reaction to occur for only a short time, did not carry the equilibrium as far toward neutrality as nature did in a much longer time. But even after the longest period of time, an acidity seemed to have developed which was sufficiently reactive to be measured by the method. This apparent inconsistency means only that there is probably always a slowly reactive acidity in a soil, due to acid silicates, organic substances or other amphoteric colloids.

5. The growth of alfalfa was at a maximum when an amount of lime approximately equivalent to the indicated requirement was added. Growth was nearly as good without any lime as with the heavier applications.

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September, 1920 Research Bulletin No. 61

A STUDY OF LACTOSE-FERMENTING YEASTS PRESENT IN "YEASTY" CREAM

BY B. W. HAMMER AND W. A. CORDES

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A Study of Lactose-fermenting Yeasts Present in "Yeasty" Cream

By B. W. Hammer and W. A. Cordes

The shipment for considerable distances of cream to be made into butter, either from receiving stations or from individual farmers, is a common practice in many sections. Much of this cream is sent without refrigeration and undergoes extensive changes as a result of the activity of the organisms contained. The type of fermentation that occurs is influenced to a considerable extent by the types of organisms present, but other factors, the most important of which is apparently the temperature, cause a significant effect. One of the important fermentations occurring in cream during the warm months is the "yeasty" or "foamy" fermentation.

Typical "yeasty" or "foamy" cream has a definite yeasty odor and shows much gas formation. Cans in transit often have part of the cream forced out by the gas development. In the receiving rooms of the large creameries the can covers are commonly thrown violently when the seals are broken and the cream then foams out of the cans. The actual loss of cream is of importance, but in addition to this there is a deterioration in the quality of the cream as a result of the development of the responsible organisms. "Yeasty" cream cannot be made into the best quality of butter, since the characteristic odor and flavor are carried over into the finished product. The presence of acid apparently has no restraining influence on the development of the typical "foamy" condition. The "yeasty" fermentation of cream is accordingly of importance from the standpoint of the transportation of sour cream.

From a practical consideration, the object of a study of the "yeasty" fermentation is its elimination or control. Accomplishment of this object is dependent upon knowledge, first, whether or not the fermentation is always due to the same organism, and second, whether or not the organisms causing this change are characteristic of "yeasty" cream alone, or are present in cream, milk and other materials that do not show this condition. The work herein reported was carried out in the hope of supplying information on these two points.

HISTORICAL

The odor of "yeasty" or "foamy" cream is very suggestive of yeasts and the prevalent idea, even among those who have given the subject no particular attention, is that yeasts are the

responsible organisms. Since gas is evolved in such quantities in the typical fermentation, it is quite logical to assume that the yeasts are lactose-fermenters.

In a study of "yeasty" cream, Hunter (11) examined numerous samples from different parts of Kansas and found the predominating type of organism to be a lactose-fermenting yeast. He says: "Raw or sterile cream inoculated with a pure culture of the yeast shows typical foaming characteristics."

Lactose-fermenting yeasts have been found to be the cause of an abnormal condition in cheese in a number of instances. In his studies on Italian cheese, Bochicchio (3) found a yeast capable of fermenting lactose with the production of CO_2, responsible for the undesirable swelling. He proposed the name *Lactomyces inflans casei-grana* for the organism. It coagulated milk in a few days and partial proteolysis followed. Hard cheese made from milk inoculated with a culture of the yeast developed a "huffed" appearance, with large holes in the surface layers. In whey, the yeast produced a foaming, pleasant-tasting beverage.

Harrison (8) described under the name of *Torula amara* a lactose-fermenting yeast causing a bitter taste in milk and cheese. The bitter taste was detected in milk after five or six hours at 37° C., and after fourteen hours the taste and smell were disagreeable and strong; after ten days the milk was slightly thickened and slightly acid. The organism was found associated with the leaves of a certain species of maple near which the milk cans of a factory having trouble with the bitterness were kept.

Russell and Hastings (18) found a lactose-fermenting yeast as the cause of a gassy fermentation in Swiss cheese. They found that in the factory experiencing the trouble, ideal conditions existed for the growth of the organism, since the whey was held in wooden vats for twenty-four hours to allow creaming of the fat. The cream skimmed from these vats had a decidedly yeasty odor and contained a lactose-fermenting yeast, whose development was favored by the strongly acid reaction of the whey. During the season over two tons of cheese were utterly ruined and a loss of over $2000 incurred by the one factory. In a later investigation Hastings (9) found that lactose-fermenting yeasts were widely distributed in such products as butter, milk, cheese, whey, and rennet.

In addition to the lactose-fermenting yeasts already mentioned as responsible for rather extensive abnormal conditions in dairy products, many others have been isolated from various sources and described. Dombrowski (4) has recently reviewed these in an extensive way and the following is in part taken from his report.

The first budding fungus able to break up lactose into alcohol and CO_2 was found in milk in 1887 by Duclaux (5). Its small size distinguished it from the yeasts known to the fermentation industries.

In 1889 Adametz (1) isolated from milk and described a new lactose-fermenting yeast which he named *Saccharomyces lactis*. This organism and *Saccharomyces tyrocola*, which was repeatedly encountered in Edam cheese by Beyerinck (2), have been exhaustively studied by Heinze and Cohn (10). *Saech. tyrocola* was found to produce a much quicker and more energetic fermentation in milk, to prefer a lower temperature, and to be less resistant to heat than *Saech. lactis*. Neither of the organisms was shown to produce spores, and Heinze and Cohn suggested that the names be changed to *Torula lactis* Adametz and *Torula tyrocola* Beyerinck. The latter organism and *Torula kefir* (isolated from kefir grains) were supposed, according to Beyerinck, to hydrolize lactose and sucrose, but not maltose.

Heinze and Cohn observed that most of the lactose-fermenting yeasts were torulae, and that several organisms belonging to the mold fungi also possessed the ability to ferment lactose. They referred to two yeast-like mold fungi which Lindner called lactose-fermenters, altho the lactose fermenting ability of one of these forms, *Monilia variabilis*, had not been determined absolutely. This yeast-like organism was first found by Lindner on damp white bread. The second organism Lindner called *Sachsia suaveolus;* it was supposed to produce an exceptional "bouquet" in wort.

Macé (14) demonstrated that the French soft cheeses very commonly contain lactose-fermenting yeasts. He found in all eleven lactose-fermenting yeasts, ten of which were torulae, in eight out of thirteen samples of this type of cheese.

Two lactose-fermenting yeasts were found by Adametz and Winkler (20) in Olmützer "quargel" cheese; one of these organisms, which gave a yellow-green fluorescent pigment on gelatine, produced CO_2 from sugars, but no alcohol.

Nikolajewa (15) isolated two torulae from kefir grains, one of which fermented lactose, sucrose, and dextrose. These forms were supposed to produce the coat of the grain.

Among the lactose-fermenting yeasts producing spores (true Saccharomycetes) the following are of importance:

(1) A yeast isolated by Grotenfelt (6) from a sample of Finland milk was considered by Hansen to be a true Saccharomycete and was named *Saccharomyces acidi lactici* by Grotenfelt. This form produced in sterile milk a weak alcoholic fermentation and a large amount of acid, which coagulated the milk.

(2) In 1890 Weigmann (21) isolated a lactose-fermenting Saccharomycete from a sample of deteriorated butter.

(3) *Saccharomyces fragilis* n. sp. Jörgensen (12) was found in kefir; the cells of this form possessed a characteristic power of weak refraction of light. Alcohol and also acid were produced from lactose by this yeast.

(4) In 1897 von Freudenreich and Orla-Jensen (19) found a true Saccharomycete in their work on the influence of natural rennin upon the ripening of Swiss cheese. The authors could not identify this yeast as one of the described forms. The yeast was not found in the natural rennin, as it was destroyed by the heat used in the preparation, but was found to be present in the so-called "acid" used in making the rennin.

(5) In 1902 Orla-Jensen (16) found two lactose-fermenting true Saccharomycetes in his investigation on the production of rancidity in butter. These yeasts fermented maltose as well as lactose. Inoculated butter remained unchanged even after two months; the acid number did not increase, as the yeast lacked the power to split fat.

(6) In 1903 Macé (14) reported finding a lactose-fermenting true Saccharomycete in his work on the soft cheeses of France.

(7) In 1908 Weigmann, Gruber and Husz (22) reported a Saccharomycete, encountered in their work on Armenian matzun, which they called *Saccharomyces Pastorianus*.

(8) In the same year Kuntze (13) found a lactose-fermenting yeast, a true Saccharomycete, to be constantly present in yughourt, to which it gave, by reason of its "aroma building" power, a highly desirable ester-like flavor. Milk was fermented with the production of a fine foam and an aroma suggestive of dried fruit.

The original work conducted by Dombrowski (4) concerned itself mainly with cultures furnished by others. These had their origin in milk beverages, such as matzun, kefir, and yughourt, in kefir grains, in milk, butter and butter cultures. It was found that Torula species were much more commonly met with than true Saccharomycetes. The latter were divided as follows:

1. Genus Saccharomyces.
   a. *Saccb. lactis alpha.*
   b. *Saccb. lactis beta.*
   c. *Saccb. lactis gamma.*
2. Genus Zygosaccharomyces, represented by one species, *Z. lactis alpha.*

Out of six cultures of true Saccharomyces worked with, four (comprising the alpha and beta species) fermented dextrose, galactose, lactose and sucrose, but not maltose. The other two cultures (*Saccb. lactis gamma*) fermented all the sugars but maltose and lactose. The one species of Zygosaccharomyces was isolated from butter, and was found to ferment dextrose, galactose, lactose, and sucrose, but not maltose, producing besides alcohol and

CO_2, a small amount of acid. This species showed a fusion of the cells before spore production. It was supposed to be the first lactose-fermenting yeast of the genus to be described.

Of thirteen cultures of torula forms worked with by Dombrowski, six fermented lactose with the production of CO_2, and the others fermented no sugars. These torula forms were isolated from matzun, kefir, and kefir grains, milk and butter.

Rubinsky (17) described a lactose-fermenting yeast which he isolated from kumiss. Spore production was not certain. Milk held at 37° C. for several days coagulated, following an evolution of CO_2. This coagulation was attributed to an enzyme. The yeast was supposed to differ from *Saccharomyces kefir* Beyerinck in its ability to coagulate milk and produce peptone.

EXPERIMENTAL

METHODS USED

The samples of milk, cream, etc., were examined for lactose-fermenting yeasts by plating on whey agar and incubating at 37° C., 1 cc. of a 1 per cent solution of tartaric acid being added to each plate just before pouring. Yeast colonies are easily detected on such plates and the low power of the microscope affords a simple means of making a decision in case of doubt. The different types of yeast colonies which developed on the plates were inoculated into milk and all those developing gas were reserved for study.

Enrichment methods were commonly used, especially if difficulty was encountered in securing a lactose-fermenting yeast from the material under study. These methods consisted of one or more transfers in milk or lactose bouillon, incubation being at 37° C.; plating was then carried out in the usual way.

Purification was accomplished by repeated plating. In some instances difficulty was experienced in securing pure cultures and bacteria were still present after several platings; the almost constant presence of bacteria in cultures of certain yeasts suggested a tendency of these organisms to attach themselves to the yeast cells.

The acidity of fermented milk was determined by titrating a 5 or 10 cc. portion with N 10 NaOH, using phenolphthalein as an indicator; the results were expressed as the percentage of lactic acid. If there was active gas evolution a 10 gram sample was used.

The amount of alcohol produced was estimated by distilling, after the addition of an excess of $CaCO_3$, and determining the specific gravity of the distillate. NaCl was usually added in considerable quantities and foaming was controlled with tannic acid.

Spore production was determined by the gypsum block method. After a number of transfers in glucose bouillon or on whey agar the cells were washed twice with sterile distilled water, using a centrifuge, and put on blocks; stains for spores were made after varying periods. Old agar cultures (from one to four months old) were also examined for spores, since tests with known spore formers showed that spores were readily detected in such cultures; if results are not wanted quickly this method has the advantage of involving much less work than the block method.

Giant colonies were secured by putting a small drop of a sugar bouillon culture on a whey gelatine plate and incubating at 20° C.

RESULTS SECURED

Samples of "yeasty" or "foamy" cream examined.

Twenty-one samples of definitely "yeasty" cream were examined; some of these were collected by a laboratory representative, while others were sent in on request, so the decision as to whether or not the samples were "yeasty" rested with a number of different people. The material was intentionally secured from rather widely separated sections so as to represent variable conditions. The number of samples of "yeasty" cream from each state was as follows: Iowa, 13; Kansas, 4; Missouri, 2; Illinois, 1; and Nebraska, 1.

From each of the 21 samples, a lactose-fermenting yeast was readily isolated without the use of enrichment methods. The organisms secured from twenty of the samples were of the same general type as regards morphology and the common growth characteristics; this type is referred to as Type A. Another type of organism, Type B, was secured from one of the samples from which Type A could not be isolated. In all of the samples of "yeasty" cream examined, the lactose-fermenting yeasts were present in considerable numbers.

Samples of number 2 cream examined.

Nine samples of number 2 cream were secured from Iowa creameries and examined for lactose-fermenting yeasts. The samples were taken from lots of cream that had been graded out by the cream graders and represented cream of a very poor quality. None of the samples showed any evidence of a "yeasty" condition.

Lactose-fermenting yeasts were isolated from eight (89 per cent) of the nine samples examined; from seven of the eight positive samples Type A was secured and the remaining one yielded Type B as the only lactose-fermenting yeast. The organisms were easily isolated, altho with a few of the samples enrichment methods were employed.

The results secured are of importance mainly in that they indicate that lactose-fermenting yeasts were commonly present in

samples of number 2 cream which showed no evidence of a "yeasty" condition.

Samples of clean-flavored sour cream examined.

Seventy-one samples of clean-flavored sour cream coming from various parts of Iowa were examined for the presence of lactose-fermenting yeasts. The common procedure was to hold the cream at 37° C. for from 24 to 48 hours and then plate in the usual way. With this method, yeasts were secured from all the samples examined, but only twelve of the 71 samples (17 per-cent) yielded lactose-fermenting types. Of the twelve positive samples, eleven yielded lactose-fermenters of the A type and one a lactose-fermenter of the B type.

The data presented show that lactose-fermenting yeasts were sometimes present in clean-flavored samples of sour cream, but that they were much less common than in the small number of samples of number 2 cream examined.

Presence of lactose-fermenting yeasts in milk.

Lactose-fermenting yeasts have been frequently met with in samples of milk from various parts of Iowa held at 37° C. for the purpose of isolating other types of organisms. When the milk was held in a tightly corked bottle it was very common to find the cork blown out and in some instances the bottle broken. No data were secured on the percentage of the samples of milk containing lactose-fermenting yeasts.

Lactose-fermenting yeasts were isolated from two samples of Iowa milk and also from a sample of milk sent from Denmark; these were of the A type. The isolations from milk were made by plating the milk after it had shown gas development.

A lactose-fermenting yeast of the A type was also secured from a sample of matzum. Altho the matzum had been carried for a considerable time in the United States, the original material was brought from Armenia; it is of course impossible to tell whether or not the yeast was a contamination picked up in this country.

STUDY OF THE LACTOSE-FERMENTING YEASTS

The 45 lactose-fermenting yeasts isolated from the samples of cream, both "yeasty" and not "yeasty," and from the samples of milk, were studied for the purpose of comparing them. The sources of these cultures are summarized in table I.

A careful study of the morphological, cultural and bio-chemical features of the 45 cultures led to their division into two types, Type A and Type B, as already mentioned, that are apparently quite distinct. Altho only three cultures of Type B were studied, because it is apparently rather unusual, these three cultures were so alike and so definitely different from the other cultures that their separation seemed justifiable. The main differences between the two types were as follows:

TABLE 1—SOURCES OF LACTOSE-FERMENTING YEASTS STUDIED

Material used for isolation	Origin by States and Countries	No. of Cultures
"Yeasty" cream	Ia., Kan., Mo., Ill., Neb.	21
Number 2 cream	Ia.	8
Clean-flavored sour cream	Ia.	12
Milk (including matzun)	Ia., Denmark, possibly Armenia	4
	Total	45

1. Type A was consistently larger than Type B.

2. Type A was oval or elliptical with elongated cells frequently present in certain cultures, while Type B, in the few cultures studied was always almost spherical.

3. In general Type A was a more rapid grower than Type B. While the optimum temperature for development of Type A was about 37° C. and that for Type B about 30° C., whey agar slope cultures of the former showed better development at 37° C. than did those of the latter at 30° C. Even when compared at the optimum temperature for Type B, whey agar slope cultures showed Type A to develop slightly better. At 37° C. the better development of Type A was marked. The same relationship was found in a comparison of whey agar plate colonies. After 24 hours at 37° C., Type A developed colonies about 2.5 mm. in diameter, while Type B, at its optimum temperature of 30° C., did not exhibit colonies of this size until after 48 hours. Type A showed a better development of colonies than Type B even at the optimum temperature of the latter. The more rapid growth of Type A was also shown by the rate of gas formation in various media containing a fermentable carbohydrate. The gas formed by a number of cultures in fermentation tubes containing sterile milk and whey after varying periods at 37° C. is shown in table II. The more rapid growth of Type A in these trials was due to the two factors mentioned above — first, the optimum for Type B was at a lower temperature than that for Type A, and second, Type A was in general a more rapid grower than Type B, even when both organisms were close to their optima.

4. All of the three cultures of Type B studied were true gelatine liquefiers, while true liquefaction was rare with Type A. The most of the cultures of Type A, however, changed the gelatine to a liquid condition at 20° C. but solidification took place at somewhat lower temperatures. When a series of Type A cultures was cooled below 20° C., a setting of the gelatine occurred first in one tube and then in another, until at about 8° C. most of them were solid. Cultures solidifying when cooled to about 8° C. were not considered true liquefiers, since cultures of Type B and known gelatine liquefiers did not do this. The reason for the peculiar behavior of cultures of Type A was at first thought to be due to the presence of alcohol, which is said to influence

TABLE II—RATE OF GAS FORMATION BY TYPES A AND B IN STERILE MILK AND WHEY IN FERMENTATION TUBES AT 37° C.

MILK

Culture No.	Type	Percent Gas after Various Periods								
		31 hr.	40 hr.	45 hr.	64 hr.	73 hr.	88 hr.	191 hr.	120 hr.	136 hr.	161 hr.
115	A	10	20	42	72	75	80	82	max.	...	...
142	A	trace	35	45	68	85	90	max.	...	...	...
179	A	trace	95	100	...	...	...	...	...	...	...
182	A	0	40	78	90	100	...	...	...	...	...
69	B	0	0	2	90	100	...	...	...	...	...
168-2	B	0	0	0	51	61	90	100	...	...	...
174-1	B	0	0	trace	61	68	80	85	85	98	100

NEUTRAL WHEY

Culture No.	Type	Percent Gas after Various Periods							
		41 hr.	53 hr.	65 hr.	73 hr.	88 hr.	113 hr.	136 hr.	168 hr.
22	A	0	trace	1	5	30	75	max.	...
120	A	0	20	56	70	78	84	max.	...
147	A	20	80	85	max.	...	...	...	...
179	A	20	54	82	92	100	...	...	...
69	B	0	0	0	0	70	80	100	...
168-2	B	0	0	0	0	14	36	48	max.
174-1	B	0	0	0	0	25	78	max.	...

the solidifying properties of gelatine, but the addition of alcohol to uninoculated gelatine tubes failed to reproduce the condition.

5. Types A and B differed in their ability to ferment various materials. Both fermented fructose, galactose, glucose, lactose, and sucrose, but neither fermented glycerol, maltose, dulcitol, mannitol, or salicin. Type A usually fermented inulin and raffinose, while the three cultures of Type B fermented raffinose, but not inulin. The differences in the fermenting powers of the two types are shown in the following summary.

I. Fermenting inulin.
   a. Fermenting raffinose. — 37 cultures Type A
   b. Not fermenting raffinose. — 3 cultures Type A

II. Not fermenting inulin.
   a. Fermenting raffinose. — 3 cultures Type B
   b. Not fermenting raffinose. — 2 cultures Type A

6. Type B was not able to grow at 43° C., while some cultures of Type A grew at this temperature and some did not.

7. Some cultures of Type A exhibited a "radial thread" subsurface colony in whey agar plates, somewhat similar in appearance to a mold colony. Type B showed only dense sub-surface colonies, with entire, well-defined edges.

Designation of the organisms studied.

The two types of lactose-fermenting yeasts were studied morphologically, culturally and bio-chemically and the descriptions, which are given below, have been compared with those of the torulae reported as having been isolated from milk and other dairy products; both the original reports and descriptions given

by Guilliermond (7) have been used. Many of the organisms have been so inadequately described that it is entirely impossible to say whether or not the organisms studied are like them. The problem is further complicated by the fact that the lactose-fermenters often coagulated milk very slowly and it is likely that organisms have been erroneously reported as not coagulating milk because no particular attention was given to this point. An attempt was made to use the names of organisms already described for the types studied, but this was found to be impossible, since the named organisms that were the most like these always showed differences that it seemed could not logically be disregarded. Accordingly, *Torula cremoris* is proposed for Type A and *Torula sphaerica* for Type B.

DESCRIPTION OF TYPE A—TORULA CREMORIS

MORPHOLOGY.

Form. In general the cells were oval or elliptical. Some cultures exhibited cylindrical and elongated cells; there was a certain variation in all cultures, but it was more pronounced in some than in others.

Size. In 24 hour whey agar slope cultures the organisms varied generally from about 2.5 to 3.6 microns in width and from about 4.1 to 6.1 microns in length. Some cultures showed elongated cells measuring usually about 3 to 4 by 13 to 14 microns altho cells as long as 19 microns were not uncommon.

Arrangement. The organisms commonly occurred singly; in young cultures a single bud was usually attached to the mother cell.

Spore Formation. The organisms were not observed to form spores, altho the most favorable conditions known for spore formation were employed.

Staining Reactions. The cells stained readily with the ordinary stains. They were Gram positive in young cultures, but in old cultures some Gram negative cells were observed.

CULTURAL CHARACTERISTICS.

Whey Agar Slope. After 24 hours at 37° C., a good development was evident. The growth was generally raised, glistening, non-viscous, whitish, smooth and opaque, the edge being entire, or slightly undulate. With age the amount of growth increased but the general character was retained. At room temperature the same type of growth was secured, but the development was not so rapid.

Beef Infusion Agar Slope. Growth was slow, only fair in amount, and apparently slightly better at 20° C. than at 37° C. It resembled in general characteristics that secured on whey agar.

Whey Agar Stab. After 24 hours at 37° C. there was a very heavy, white, glistening, non-viscous surface growth considerably raised and with an entire edge. There was a heavy growth all along the line of inoculation, altho development was best near the surface. After several days some cultures exhibited a suggestion of a villous character of growth, but with very short, thread-like projections along the stab. At room temperature the growth was of essentially the same character as at 37° C., but was less rapid.

Beef Infusion Agar Stab. Growth was slow and never abundant, but resembled in general characteristics the growth secured on whey agar.

Whey Agar Plate Colony. Colonies developed rapidly at 37° C. After 24 hours the surface colonies were usually large, averaging about 2.5

mm. in diameter, regularly circular, glistening, non-viscous, whitish, convex, smooth, and opaque, with an entire edge. Under the microscope the surface colonies appeared dense, with the typical edge of closely packed yeast cells. With some cultures the sub-surface colonies were consistently of the "radial thread" type, presenting an appearance similar to the hyphae of a mold colony; with other cultures they were dense, oval, spherical, or "torpedo-like," exhibiting an entire, definite edge. There were all gradations evident between these two extreme types of sub-surface colonies, but individual cultures were constant in the type of sub-surface colony developed. Growth at room temperature was the same as at 37° C. but was slower.

Giant Colony. After 18 days on whey gelatine, giant colonies measured usually about 9.5 to 13 mm. in diameter, were grayish, raised, slightly glistening, opaque, with a depressed, smooth center surrounded by a raised ring, from which radial contours extended to the edge. A softening of the gelatine took place usually after a number of days, the colonies sinking into the medium.

Whey Gelatine Slope. Growth was rather slow, but after six days a fair amount was present; it was filiform, whitish gray to cream colored, dull or slightly glistening, somewhat raised, with transverse contours, while the edge was straight or undulate. In some cultures a softening of the gelatine occurred in about two weeks.

Whey Gelatine Stab. Growth was slow, but developed well. After six days there was a fair amount of surface growth that was raised, circular, whitish gray to cream colored, dull, or slightly glistening, with radial contours. Along the line of the stab the growth was especially heavy just below the surface. After from two weeks to a month, a softening of the gelatine took place in some cultures, a few showed a true liquefaction, while still others exhibited no change of this nature, even when held 79 days after inoculation.

Bouillons. Growth in bouillons was evident in 24 hours at 37° C. as a whitish sediment. It was best in bouillons to which had been added materials that were fermented with the production of gas, but also occurred in plain bouillon and bouillons with non-fermentable materials. Surface growth was never observed. At room temperature growth was slower than at 37° C.

Potato. A luxuriant growth, varying in character slightly with the moisture content of the potato, was present after 24 hours at 20° C. In general the growth was slightly raised, whitish, glistening, and smooth.

Dunham's Solution. Growth was evident as a whitish sediment after several days at 37° C.

Uschinsky's Solution. Growth was evident as a whitish sediment after several days at 37° C.

Litmus Milk. After 24 hours at 37° C. much gas was formed and a small amount of acid; the litmus turned a definite pink thruout the tube. Coagulation took place after a number of days thru the agency of a rennin-like enzyme.

Plain Milk. After 24 hours at 37° C. gas evolution was pronounced. Coagulation took place after a number of days at 37° C.

BIO-CHEMICAL FEATURES.

Gas Production. Gas was produced in milk and in bouillons containing fructose, galactose, glucose, lactose and sucrose. The great majority of the cultures worked with also produced gas in inulin and raffinose bouillons, but three cultures failed to ferment raffinose and two did not ferment either inulin or raffinose. Gas production varied in amount with individual cultures, some fermenting only weakly. Bouillons containing

glycerol, maltose, dulcitol, mannitol, or salicin were not fermented with gas evolution by any of the cultures. Beer wort was commonly fermented with gas production, due probably to the presence of glucose.

Reaction Change. Sugar bouillons in which gas was produced were slightly acid in reaction, showing, on an average, from 1.6 to 1.9 percent N/1 acid. A small amount of acid was also produced in milk.

Odor. Milk and sugar bouillons fermented with gas production gave a marked odor of alcohol, the odor being especially noticeable in milk. Agar plates emitted a pleasing, fruity odor.

Alcohol Production. In milk incubated at 37° C. for several days, alcohol was produced to the extent of 1.5 to 2.0 percent by weight.

Optimum Temperature. The optimum temperature for growth was found to be about 37° C.

Oxygen Relation. The organism was facultative, but grew best in the presence of a plentiful supply of oxygen.

DESCRIPTION OF TYPE B — TORULA SPHAERICA

MORPHOLOGY.

Form. The cells were generally almost spherical; definitely elongated cells were never observed.

Size. In 24 hour whey agar slope cultures, the cells averaged from about 3.0 to 3.1 by 3.0 to 3.3 microns, most of them being almost spherical.

Arrangement. The organisms commonly occurred singly, or with a single bud attached.

Spore Formation. Spores were not observed, even under the most favorable conditions known for spore formation.

Staining Reactions. The cells stained readily with the ordinary stains. In young cultures the organisms were Gram positive, but in old cultures some Gram negative cells were seen.

CULTURAL CHARACTERISTICS.

Whey Agar Slope. After 24 hours at 37° C., a raised, glistening, whitish, smooth growth, moderate in amount, was evident; the edge was entire or slightly undulate. At 30° C. and at room temperature the growth was of the same type, but development was somewhat faster than at 37° C.

Beef Infusion Agar Slope. Growth was generally slow and only fair in amount, but possessed the same general characteristics as that on whey agar. Development was better at room temperature and 30° C. than at 37° C.

Whey Agar Stab. In 24 hours at 37° C. a fair amount of surface growth was present, which was circular, slightly convex, whitish, glistening, smooth and opaque, with an entire edge. Along the line of inoculation a heavy filiform growth occurred that was best near the surface.

Beef Infusion Agar Stab. Growth was slow and never heavy but resembled in its general characteristics that secured on whey agar.

Whey Agar Plate Colony. In 24 hours at 37° C. the colonies were barely, if at all, visible. Plates held at 30° C. for the same length of time showed rather small surface colonies; in 48 hours these developed into colonies about 2.0 to 2.5 mm. in diameter that were regularly circular, whitish, glistening, convex, smooth, and opaque, with an entire edge. After 48 hours at 37° C. the colonies were definite but very small. Subsurface colonies always had an entire edge, and were generally spherical or oval in shape; no radiating filaments were ever present in the subsurface colonies.

Giant Colony. After 18 days on whey gelatine, giant colonies measured from 10 to 12 mm. in diameter and were generally yellowish or whitish,

raised, and dull. The center appeared deeply depressed and there were fine radial contours extending from the raised ring surrounding the center to the edge. The colonies were in general smooth, with entire, regular edges.

Whey Gelatine Slope. In six days a filiform growth, whitish gray to light cream in color, was evident. A uniform depression, like a furrow, extended in the center along the length of the streak. The growth was raised and slightly glistening, some of the cultures exhibiting cross contours while others were smooth. A softening of the gelatine began in some cases in fourteen days.

Whey Gelatine Stab. In six days a good amount of surface growth was evident; it was whitish gray or cream colored, smooth, slightly raised, glistening, and had an entire edge and radial contours. Along the line of the stab the growth was filiform and heaviest just below the surface. True liquefaction took place in about one month, but before this the cultures passed thru a softening stage in which solidification took place upon cooling to 8° C.

Bouillons. Growth in bouillons was evident as a whitish sediment in from 24 to 48 hours at 37° C. It was best in bouillons to which had been added materials that were fermented with the production of gas, but also occurred in plain bouillon and bouillons containing non-fermentable materials. Surface growth did not occur. Growth was more rapid at 30° C. than at 37° C.

Potato. A heavy growth, varying somewhat in character with the amount of moisture in the potato, took place after 24 hours at 20° C. Usually the growth was whitish, glistening, smooth, and only very slightly raised.

Dunham's Solution. Growth was evident as a whitish sediment after several days at 37° C.

Uschinsky's Solution. Growth was evident as a whitish sediment after several days at 37° C.

Litmus Milk. Very slight gas production occurred in 24 hours at 37° C.; a small amount of acid was produced, the litmus turning pink thruout the tube. Gas production was more marked at 30° C. Coagulation took place after a number of days at 37° C. thru the agency of a rennin-like enzyme.

Plain Milk. Very slight gas production occurred in 24 hours at 37° C. Coagulation occurred after a number of days at this temperature.

BIO-CHEMICAL FEATURES.

Gas Production. Gas was produced in milk and in bouillons containing fructose, galactose, glucose, lactose, sucrose, and raffinose. Gas production was stronger with some of these materials than with others. None of the cultures was able to produce gas in bouillons containing glycerol, maltose, dulcitol, mannitol, salicin, or inulin.

Reaction Change. Sugar bouillons in which gas was produced were slightly acid in reaction, showing on an average, from 1.4 to 1.8 per cent N/1 acid. A small amount of acid was also produced in milk.

Odor. Agar plates emitted a pleasant, fruity odor. In milk and sugar bouillons fermented with gas production there was a marked odor of alcohol.

Alcohol Production. In milk incubated at 37° C. for a number of days, about 1.8 percent of alcohol by weight was produced.

Optimum Temperature. The optimum temperature for growth was found to be about 30° C.

Oxygen Relation. The organism was facultative, but grew best in the presence of an abundant supply of oxygen.

TABLE III—ACIDITIES (CALCULATED AS LACTIC ACID) IN SKIM MILK AT THE TIME OF COAGULATION

Culture No.	Type	Temp. of Incubation	Days to Coagulate	Acidity
69	B	37° C.	7	0.31%
174-1	B	37° C.	11	.40
174-1	B	30° C.	24	.23
168-2	B	30° C.	27	.34
147	A	43° C.	14	.33
147	A	20° C.	58	.38
39	A	37° C.	4	.31
178	A	37° C.	4	.27
179	A	37° C.	4	.36
182	A	37° C.	4	.26

TABLE IV—ACIDITIES (CALCULATED AS LACTIC ACID) IN STERILE SKIM MILK AFTER VARYING PERIODS AT DIFFERENT TEMPERATURES

Culture No.	Type	Temperature	24 hrs.	48 hrs.	3 days	4 days	5 days	7 days	8 days	14 days	21 days
22	A	37° C.		.50%		.48%		.41%			
28	A	20° C.			.55%		.54%		.50%	.34%	
		25° C.			.52		.50		.37	.38	
		37° C.		.50		.42		.38			
39	A	20° C.	.21%		.45		.58		.36	.32	.35
		37° C.		.47		.34		.42			
43	A	20° C.			.39		.59		.42	.35	.36
		37° C.		.51		.35		.38			
47	A	20° C.	.23		.49		.56		.44	.37	.39
69	B	20° C.		.46	.52		.59		.40	.35	.36
		25° C.			.57		.50		.38	.33	
		37° C.		.45		.39		.44			
70	A	20° C.			.49		.54		.43	.32	.37
		37° C.		.50		.43		.37			
80	A	20° C.			.50		.56		.42	.36	.40
		37° C.		.50		.36		.39			
120	A	20° C.	.24		.45		.52		.55	.37	.39
		25° C.			.46		.50		.35	.37	
		37° C.		.48		.50		.39			
143	A	20° C.	.28		.54		.55		.42		
		37° C.		.52		.49		.38			
147	A	20° C.	.23		.49		.59		.36	.35	.35
		25° C.			.50		.54		.36	.34	
		37° C.		.55		.47		.37			
158	A	20° C.			.48		.55		.49	.37	
		25° C.			.52		.53		.34	.35	
		37° C.		.52		.37		.38			
159	A	20° C.			.49		.54		.48	.36	.36
		25° C.			.56		.47		.33	.33	
		37° C.		.46		.42		.38			
168-2	B	20° C.			.50		.56		.42	.36	.37
		25° C.			.53		.53		.34	.31	
		37° C.		.37		.38		.40			
170	A	20° C.		.42	.47		.52		.48	.34	.32
		37° C.		.49		.56		.40			
174-1	B	20° C.			.47		.59		.50	.39	.41
		25° C.			.45		.54		.33	.29	
176	A	37° C.		.51		.48		.40			
177	A	37° C.		.47		.36		.37			
179	A	37° C.		.48		.40		.41			
183	A	37° C.		.48		.35		.38			

ADDITIONAL DESCRIPTION OF CHARACTERISTICS OF YEASTS ISOLATED

All of the lactose-fermenting yeasts studied curdled milk and the coagulation was not due to the acid, as is shown by table III, which gives the acidities present in milk at the time of coagulation after inoculation with various cultures. The data show that coagulation occurred at acidities varying from 0.23 to 0.40 per cent and accordingly it must be assumed that a rennin-like enzyme was responsible for the curdling.

Higher acidities were observed soon after inoculation than later on, as is shown in table IV; this was undoubtedly due to the presence of much larger quantities of CO_2 during the period of active gas formation than later when gas evolution was no longer evident. Table V gives the final acidities produced by various milk cultures and the results presented show that the final acidity reached by most cultures was not sufficient to cause the coagulation of milk.

The rate of coagulation was materially influenced by various conditions. A temperature of 37° C. was satisfactory and coagulation took place here much more rapidly than at room temperature, 30° C., or 43° C.; table VI gives results showing, in a general way, the length of time required for curdling at different temperatures. At all the temperatures tried there was a great deal of variation in the time required for curdling by different cultures and also by the same culture in different tests.

TABLE V MAXIMUM ACIDITY (CALCULATED AS LACTIC ACID) PRODUCED IN STERILE SKIM MILK

Culture No.	Type	Incubation Days at 37° C.	Acidity	Days to Coagulate
22	A	33	.45	5
39	A	28	.40	8
40	A	28	.42	3
42	A	28	.38	4
43	A	33	.43	5
44	A	28	.45	2
53	A	28	.43	3
76	A	28	.38	1
78	A	28	.54	5
80	A	28	.45	8
96	A	28	.44	3
105	A	33	.46	4
111	A	33	.45	1
115	A	33	.41	4
120	A	33	.50	4
143	A	28	.40	5
144	A	28	.42	5
147	A	33	.43	5
156	A	12	.41	3
157	A	28	.29	4
158	A	28	.43	3
159	A	28	.47	4
160	A	28	.40	4
162	A	28	.40	4
163-1	A	28	.34	4
165	A	28	.36	4
168-2	B	22	.51	20
169	A	28	.37	5
170	A	28	.40	5
171	A	28	.41	4
172	A	28	.41	4
173	A	28	.49	5
174-1	B	28	.52	12
175	A	28	.42	6
176	A	33	.46	4
179	A	28	.38	4
180	A	28	.36	4
181	A	28	.50	5
182	A	28	.50	4
183	A	28	.34	4
186	A	28	.42	4

TABLE VI—TIME REQUIRED TO COAGULATE MILK AT DIFFERENT TEMPERATURES

Culture No.	Type	Temperature	Days to Curdle	Temperature	Days to Curdle	Temperature	Days to Curdle
115	A	20° C.	24	37° C.	3	43° C.	8
147	A	20° C.	58	37° C.	6	43° C.	14
69	B	20° C.	32	37° C.	7	39° C.	17
168-2	B	20° C.	not in 28	37° C.	10	36° C.	27
174-1	B	20° C.	45	37° C.	6	30° C.	24
176	A	20° C.	not in 28	34° C.	12	38° C.	4
182	A	20° C.	not in 25	37° C.	4		
71	A	20° C.	not in 25	37° C.	5		

Coagulation of the milk took place more quickly in bottles or flasks than in test tubes. In some instances a culture failed to curdle a certain tube of litmus milk, altho it caused a vigorous gas development, but transfers made to other tubes of milk showed coagulation. The length of time required for coagulation in both flasks and tubes is shown for certain cultures in table VII; no explanation for the variations could be found, altho an attempt was made to work out an explanation on the basis of such factors as air supply, presence of soluble calcium salts and the extent of heating during sterilization. Coagulation generally occurred in a shorter time in whole milk in test tubes than in skim milk in the same kind of containers. It seems probable that the long periods required for coagulation in test tubes and the actual failures to secure such a change in some cases are the reasons why certain investigators have not reported the cultures of yeasts they studied as capable of coagulating milk.

TABLE VII—COMPARISON OF FLASKS WITH TUBES IN TIME REQUIRED TO CURDLE SKIM MILK AT 37° C.

Culture No.	Type	Number Days Required to Curdle	
		In Flasks	In Test-tubes
22	A	5	12
39	A	8	11
10	A	3	26
43	A	5	16
44	A	2	14
53	A	3	14
70	A	4	16
73	A	5	14
86	A	8	19
96	A	3	6
111	A	4	20
115	A	4	15
143	A	5	26
147	A	5	11
157	A	4	11
158	A	3	16
159	A	4	11
166	A	4	16
162	A	4	22
163-1	A	4	22
170	A	5	11
180	A	4	16
183	A	4	22
186	A	4	23

From a number of experiments carried out it seems that the coagulating enzyme, while apparently produced in considerable amounts at temperatures such as 20° C., is much more active at higher temperatures. Cultures held for long periods of time at 20° C. without coagulating, showed this change in a comparatively short time at higher temperatures. For example, a culture which had been held at 20° C. for 28 days without coagulation, showed this change in 70 minutes after it had been placed at 43° C. In another instance a culture, after being held at 20° C. for 56 days without showing coagulation, showed this change in a portion of the culture held at 43° C. in about the length of time required to thoroly warm it up, while the remainder of the culture at 20° C. first showed coagulation after 58 days. Cultures held for long periods at 20° C. without coagulation showed this change when placed at 37° C., but not as quickly as at 43° C.

The alcohol produced by the lactose-fermenting yeasts was never very high in milk, the maximum percentage found being 2.28 percent by weight. Table VIII presents data showing in a general way the amount of alcohol produced in milk at 20° C., 25° C. and 37° C.; the results show that alcohol production occurred at all the three temperatures tried and that apparently 25° C. was very favorable.

Table IX gives the alcohol produced by the lactose-fermenting yeasts in bouillons containing glucose, lactose, and sucrose in 5 and 10 percent amounts, when incubated for 21 days at 37° C. The data show that under suitable conditions the yeasts can produce alcohol in excess of the amounts they produce in milk. The maximum percentage observed was 4.00 and this was produced in 10 percent lactose bouillon. The 10 percent sugar

TABLE VIII—ALCOHOL PRODUCTION IN STERILE SKIM MILK

Culture No.	Type	Time in Days	Temperature	Percent Alcohol by Weight
28	A	22	20° C.	1.94
28	A	33	25° C.	2.28
40	A	16	37° C.	1.37
163-1	A	19	20° C.	1.62
163-1	A	18	25° C.	2.28
163-1	A	24	37° C.	2.11
165	A	20	25° C.	1.75
165	A	13	37° C.	1.87
174-1	B	16	20° C.	1.50
174-1	B	16	25° C.	1.81
182	A	5	20° C.	1.06
182	A	5	25° C.	2.11
182	A	5	37° C.	2.00
182	A	11	20° C.	2.11
182	A	11	25° C.	1.81
182	A	11	37° C.	1.81

TABLE IX—ALCOHOL PRODUCTION IN SUGAR BOUILLONS

Incubation 21 days at 37° C.

Culture No.	Type	Sugar Bouillon		Percent Alcohol by Weight
		Kind	Percent Sugar	
28	A	Sucrose	5	2.06
			10	3.82
42	A	Sucrose	5	1.87
			10	3.18
69	B	Sucrose	10	3.65
115	A	Lactose	5	1.94
147	A	Lactose	5	1.91
174-1	B	Lactose	5	1.56
158	A	Lactose	10	4.00
159	A	Lactose	10	3.71
186	A	Lactose	10	3.35
168-2	B	Glucose	10	3.24
181	A	Glucose	10	3.65

bouillons always gave alcohol in excess of that produced in those containing only 5 percent.

THE PRODUCTION OF TYPICAL "YEASTY" CREAM

Altho pure cultures of the lactose-fermenting yeasts produced much gas in milk, they did not produce a typical "yeasty" condition, in which the material continued for some time to increase in volume and a portion to be forced rather slowly from the container. The bubbles of gas passed rapidly through the milk and broke at the surface so that there was no definite and decided volume change, altho there was considerable foam. With cream, which has a greater viscosity than milk, the bubbles of gas seemed to be prevented from rapidly escaping, and in this material a condition quite like that of typical "foamy" cream could be produced. Milk which had been previously coagulated by the inoculation of *S. lacticus*, for the purpose of preventing the rapid escape of gas, did not exhibit the typical "foamy" character upon introduction of the lactose-fermenting yeast. While the yeast was capable of causing coagulation in milk, this occurred only after some time, when the rapid evolution of gas had ceased. Apparently the production of the typical "yeasty" condition in cream is very intimately connected with the prevention of the escape of the bubbles of gas formed. A "yeasty" cream was produced by the inoculation of a pure culture of the yeast into sterile cream, but apparently coagulated cream, upon introduction of the yeast, approached more closely the typical "foamy" condition so commonly met with in practice, since it continued to "boil over" for a longer period of time than did the uncoagulated cream. Accordingly it seems justifiable to assume that the condition of "foamy" cream as observed under practical conditions is not the result of the action of a lactose-fermenting yeast

alone, but is produced by a combination of the yeast and organisms which are capable of causing a rapid coagulation. It is probable that the decreased solubility of gas at higher temperatures is important, in that fermenting milk or cream, when raised somewhat in temperature, as is apt to occur during the shipment of cream in the warm summer months, produces bubbles of gas more rapidly than would be the case as a result of the immediate action of the yeasts. In inoculation experiments, the transfer of cream to 37° C. after a considerable period of incubation at a lower temperature often resulted in a "foaming-over" of the material in the container. It seems, therefore, that the temperature change may be important in the production of typical "foamy" cream.

In the attempts to produce "foamy" cream, better results were secured when the containers were corked, or in the case of a tin container, closed with a cover, than when they were stoppered with cotton or left open. It seems probable that the effect here was due to the influence on the amount of gas in solution; with the tightly closed containers there was a larger amount of gas held in the cream, so that when the stopper or cover was blown out or removed, the gas quickly formed bubbles, the volume increased, and the cream foamed over.

With gas-producing yeasts, the supersaturation with gas of the medium in which they are growing is very common. This gas is ordinarily in part quickly released on agitation. The agitation of cream inoculated with lactose-fermenting yeasts seemed to have an effect on the production of a typical "foamy" condition presumably due to the liberation of quantities of gas present in excess of the amounts required for saturation. During the shipment of cream in cans and during the initial handling at the manufacturing plant, the agitation would tend to release gas present in amounts in excess of that required for saturation.

DISCUSSION OF RESULTS

From the data presented it is evident that lactose-fermenting yeasts are readily isolated from samples of "yeasty" cream and that these are largely of one type. The results obtained also show that the same type of yeast is very common in number 2 cream and fairly common in clean-flavored sour cream, while the general statements made show it is also present in milk. Another type of lactose-fermenter possessing characteristics definite enough to distinguish it from the first, is apparently much less common, having been found in only one sample of "yeasty" cream, one sample of number 2 cream and one sample of clean-flavored sour cream.

It is probable that the more rapid growth and high optimum

temperature of the first type are the characteristics responsible for its much greater prevalence.

It is evident that the yeasts responsible for "yeasty" cream are much more common than the number of samples of cream developing a "yeasty" condition would indicate, and accordingly it seems probable that whether or not a sample of cream becomes "yeasty" is largely determined by the conditions under which the cream is held. The yeasts generally responsible for "yeasty" cream grow best at a fairly high temperature and it would be expected that the greatest trouble would be experienced during the warm months. Samples of milk and some few samples of cream have been put at 37° C. during the cold months and the development of gas by yeasts has been commonly observed; this indicates that the lactose-fermenters are also present in milk and cream during the colder weather, and in conformity with this an occasional batch of "yeasty" cream is encountered during the winter months, especially in lots that have been held at a fairly high temperature. The evidence seems to show that cream is quite likely to contain lactose-fermenting yeasts capable of producing a "yeasty" fermentation under the proper conditions, but that the conditions are not generally suitable for the fermentation to occur, altho they are much more commonly so in summer than in winter.

The production of a typical "yeasty" condition is, however, influenced by other factors than the presence of lactose-fermenting yeasts and a temperature suitable for their growth. It seems that among these additional factors those of the most importance are a viscosity, or thickening due to coagulation, sufficient to prevent too rapid an escape of the gas formed, the decreased solubility of the gas at a higher temperature, the closing of the containers, and agitation. A suitable balance between all or nearly all of the influencing factors is likely necessary before a typical "yeasty" condition results.

From the standpoint of the elimination of "yeasty" cream under practical conditions, the logical thing seems to be the prevention of the growth of the yeasts. Their frequent presence in milk and cream suggests a wide distribution of the organisms and under these conditions it is inadvisable to attempt to exclude them from cream. The next possibility is to prevent their development, and according to present knowledge, this can best be accomplished for the small shipper by the use of low temperatures. In the case of larger shipments, such as those coming from cream stations, where the volume of cream justifies the expense, effective pasteurization offers another method of preventing a loss of cream or a deterioration in quality. Yeasts are comparatively non-resistant to heat and pasteurization affords a satisfactory method of destroying them.

SUMMARY

1. Lactose-fermenting yeasts were isolated from all of 21 samples of "yeasty" cream secured from five states, from eight (89 percent) of nine samples of number 2 cream obtained from Iowa, from twelve (17 per cent) of 71 samples of clean-flavored sour cream coming from Iowa, from three samples of milk (two from Iowa and one from Denmark), and from one sample of matzun.

2. The 45 cultures studied were divided into two distinct types. These types did not agree closely enough with described organisms so that they could be considered like any of them and accordingly the names *Torula cremoris* and *Torula sphaerica* are proposed.

3. The data indicate that lactose-fermenting yeasts like those causing a "yeasty" condition in cream are much more common than the number of lots of cream developing "yeastiness" would indicate. It seems evident that the conditions to which cream is usually subjected are not suitable for the development of "foaminess," altho such conditions are much more commonly fulfilled in warm weather than in cold.

4. Factors other than the presence of lactose-fermenting yeasts and a temperature suitable for their growth that seem to be important in the development of a typical "yeasty" condition in cream, are a viscosity, or thickening due to coagulation, sufficient to prevent too rapid an escape of gas, a decreased solubility of the gas when the fermenting cream is placed at a higher temperature, the closing of the containers, and agitation.

5. Because of the wide distribution of lactose-fermenting yeasts, it does not seem advisable to attempt to keep them out of cream in order to prevent losses. The best thing that can be done at present by the small shipper is to hold cream at temperatures sufficiently low so that the development of the yeasts will be prevented or greatly retarded. Losses in large shipments of cream can undoubtedly be eliminated by efficient pasteurization and this may eventually prove to be a help in the solution of the "yeasty" cream problem.

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